FORM PTO-1390 (Modified) (REV 11-2000)	U S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		29314/35410A
		U S APPLICATION NO IF KNOWN, SEE 37 CFR **10/018453**

INTERNATIONAL APPLICATION NO **PCT/EP00/03904**	INTERNATIONAL FILING DATE **30 APRIL 2000**	PRIORITY DATE CLAIMED **30 APRIL 1999**

TITLE OF INVENTION

DIAGNOSTIC SEQUENCING BY A COMBINATION OF SPECIFIC CLEAVAGE AND MASS SPECTROMETRY

APPLICANT(S) FOR DO/EO/US

Marc Zabeau and Patrick Stanssens

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below.

4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).

5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))

    a. ☒ is attached hereto (required only if not communicated by the International Bureau).

    b. ☐ has been communicated by the International Bureau.

    c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).

6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).

    a. ☐ is attached hereto.

    b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4)

7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. (c)(3))

    a. ☐ are attached hereto (required only if not communicated by the International Bureau).

    b. ☐ have been communicated by the International Bureau.

    c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.

    d. ☒ have not been made and will not be made.

8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).

9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).

10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409).

12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

14. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

15. ☐ A **FIRST** preliminary amendment.

16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.

17. ☐ A substitute specification.

18. ☐ A change of power of attorney and/or address letter.

19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.

20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).

21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).

22. ☒ Certificate of Mailing by Express Mail EL 564 460 785 US

23. ☐ Other items or information:

PCTUS1/REV03

FORM PTO-1390 (Modified)
(REV 11-2000)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER 3 0 OCT 2001
29314/35410A

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

U.S APPLICATION NO (IF KNOWN, SEE 37 CFR
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INTERNATIONAL APPLICATION NO PCT/EP00/03904	INTERNATIONAL FILING DATE 30 APRIL 2000	PRIORITY DATE CLAIMED 30 APRIL 1999

TITLE OF INVENTION

DIAGNOSTIC SEQUENCING BY A COMBINATION OF SPECIFIC CLEAVAGE AND MASS SPECTROMETRY

APPLICANT(S) FOR DO/EO/US

Marc Zabeau and Patrick Stanssens

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:
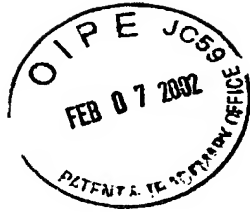
1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

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    a. ☒ is attached hereto (required only if not communicated by the International Bureau).

    b. ☐ has been communicated by the International Bureau.

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7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))

    a. ☐ are attached hereto (required only if not communicated by the International Bureau).

    b. ☐ have been communicated by the International Bureau.

    c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.

    d. ☒ have not been made and will not be made.

8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).

9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).

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13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

14. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

15. ☐ A **FIRST** preliminary amendment.

16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.

17. ☐ A substitute specification.

18. ☐ A change of power of attorney and/or address letter.

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21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).

22. ☒ Certificate of Mailing by Express Mail EL 564 460 785 US

23. ☐ Other items or information:

PCTUS1/REV03

U.S. APPLICATION NO (IF KNOWN, SEE 37 CFR	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
10/018453	PCT/EP00/03904	29314/35410A

24. The following fees are submitted·	CALCULATIONS PTO USE ONLY

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

☐ Neither international preliminary examination fee (37 CFR 1 482) nor international search fee (37 CFR 1 445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1040.00**

☒ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**

☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1 445(a)(2)) paid to USPTO **$740.00**

☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**

☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**

ENTER APPROPRIATE BASIC FEE AMOUNT =	$890.00	

Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	$0.00	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	56 - 20 =	36	x $18.00	$648.00	
Independent claims	6 - 3 =	3	x $80.00	$240.00	
Multiple Dependent Claims (check if applicable).			☒	$280.00	

TOTAL OF ABOVE CALCULATIONS =	$2,058.00	

☐ Applicant claims small entity status. See 37 CFR 1.27). The fees indicated above are reduced by 1/2.	$0.00	

SUBTOTAL =	$2,058.00	

Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +	$0.00	

TOTAL NATIONAL FEE =	$2,058.00	

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable).	☒	$40.00	

TOTAL FEES ENCLOSED =	$2,098.00	

	Amount to be: refunded	$
	charged	$

a. ☒ A check in the amount of ___**$2,098.00**___ to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. ___**13-2855**___ A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING: Information on this form may become public. Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO·

Nabeela R. McMillian
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SIGNATURE

Nabeela R. McMillian

NAME

43,363

REGISTRATION NUMBER

30 October 2001

DATE

IN THE UNITED STATES
PATENT AND TRADEMARK OFFICE

Applicants:	Zabeau *et al.*	)
		)
Serial No.:	10/018,453	)
		)
Filed:	October 30, 2001	)

Title: Diagnostic Sequencing by a
Combination of Specific Cleavage and
Mass Spectrometry

Group Art Unit: to be assigned

Examiner: to be assigned

) "EXPRESS MAIL" Mailing Label
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) indicated below and is addressed to the
) Commissioner for Patents, Washington,
) D.C. 20231, on this date,
)
) February 7, 2002
)
)
) ~~Laura Frasher~~
) Laura Frasher
)

PRELIMINARY AMENDMENT

Commissioner for Patents
Washington, D.C. 20231

Sir:

The present paper contains a preliminary amendment to be considered in the above-referenced U.S. National stage Application of PCT/EP00/03904. Applicants request that the amendment be entered and considered in the present application prior to substantive examination.

AMENDMENTS

In the claims:

Please cancel claims 1-56 and insert the following new claims:

57. [NEW] A method for sequence analysis of one or more target nucleic acids present in one or more biological samples, said method comprising the steps of:

(a)   deriving from one or more biological samples the one or more target nucleic

acids;

(b)   subjecting the one or more target nucleic acids obtained from step (a) to a set of separate base-specific, sequence-specific or site-specific complementary cleavage reactions, wherein each cleavage reaction generates a non-ordered set of fragments;

(c)   analyzing the sets of non-ordered fragments obtained from step (b) by mass spectrometry; and,

(d)   performing a systematic computational analysis on the mass spectra obtained from step (c) to analyse the sequence of said target nucleic acid,

wherein said complementary cleavage reactions refer to target nucleic acid digestions characterized by varying specificity and/or to digestion of alternative forms of the target sequence.

58. [NEW] The method according to claim 57 wherein the one or more biological samples are derived from organism selected from the group consisting of eukaryotes, prokaryotes, and viruses.

59. [NEW] The method according to claim 57 wherein the one or more target nucleic acids are selected from the group consisting of single stranded DNA, double stranded DNA, cDNA, single stranded RNA, double stranded RNA, DNA/RNA hybrid, and DNA/RNA mosaic nucleic acid.

60. [NEW] The method according to claim 57 wherein one or more target nucleic acids are derived by one or more consecutive amplification procedures selected from the group consisting of in vivo cloning, polymerase chain reaction (PCR), reverse transcription followed by the polymerase chain reaction (RT-PCR), strand displacement amplification (SDA), and transcription based processes.

61. [NEW] The method according to claim 4 wherein the one or more amplified target nucleic acids are transcripts generated from a single stranded or a double stranded target nucleic acid by a process comprising the steps of:

- 2 -

(a)   linking operatively a transcription control sequences to the one or more target nucleic acids; and

(b)   transcribing one or both strands of the one or more target nucleic acid of step a) using one or more RNA polymerases that recognize the transcription control sequence on the one or more target nucleic acids.

62. [NEW] The method according to claim 61 wherein said transcriptional control sequences are operatively linked to one or more target nucleic acids by PCR amplification using primers that incorporate the transcriptional control sequences as 5'-extensions.

63. [NEW] The method according to claim 61 wherein the transcription control sequence is selected from the group consisting of eukaryotic transcription control sequences, prokaryotic transcription control sequences, and viral transcription control sequences.

64. [NEW] The method according to claim 63 wherein the prokaryotic transcription control sequence is selected from the group consisting of T3, T7, and SP6 promoters.

65. [NEW] The method according to claim 64 wherein the RNA polymerases which utilize the T3, T7, or SP6 promoters are either wild type or mutant RNA polymerases, the mutant polymerases being capable of incorporating into the transcript non-canonical substrates with a 2'-deoxy, 2'-O-methyl, 2'-fluoro or 2'-amino substituent.

66. [NEW] The method according to claim 65 wherein the mutant RNA polymerase is either T7 or SP6 mutant polymerase.

67. [NEW] The method according to claim 57 wherein the derived target nucleic acid incorporates one or more nucleosides that are modified on the base, the sugar, and/or the phosphate moiety, wherein the modifications alter the specificity of cleavage by the one or more cleavage reagents and/or the mass and/or the length of the cleavage products.

68. [NEW] The method according to claim 67 wherein the modification is

introduced through the enzymatic incorporation of modified deoxynucleoside triphosphates, modified ribonucleoside triphosphates, and/or modified dideoxynucleoside triphosphates; or wherein the modification is introduced chemically, or wherein the modification is introduced through a combination of both methods.

69. [NEW] The method according to claim 67 wherein the modification consists of a 2'-deoxy, 2'-O-methyl, 2'-fluoro or 2'-amino substituent on the nucleotide triphosphates.

70. [NEW] The method according to claim 67 wherein the modification consists of phosphorothioate internucleoside linkages or phosphorothioate internucleoside linkages further reacted with an alkylating reagent.

71. [NEW] The method according to claim 67 wherein the modification consists of a methyl group on C5 of the uridine-5'-monophosphate subunits.

72. [NEW] The method according to claim 67 wherein the modification consists of nucleotides that incorporate alternative isotopes.

73. [NEW] The method according to claim 57 wherein the one or more target nucleic acids of step (a) are purified prior to cleavage.

74. [NEW] The method according to claim 73 wherein said purification is achieved through immobilization or by chromatography.

75. [NEW] The method according to claim 57 wherein the complementary cleavage reactions are selected from the group consisting of enzymatic cleavage, chemical cleavage, and physical cleavage.

76. [NEW] The method according to claim 75 wherein the complementary cleavage reactions are characterized by a relaxed mono-nucleotide, mono-nucleotide, relaxed di-nucleotide, or di-nucleotide specificity.

77. [NEW] The method according to claim 75 wherein the one or more target nucleic acids are subjected to chemical digestion reaction consisting of treatment with alkali or with reagents used in the Maxam & Gilbert sequencing method.

78. [NEW] The method according to claim 75 wherein the one or more target nucleic acids are subjected to enzymatic cleavage reaction using one or more enzymes selected from the group consisting of endonucleases and exonucleases.

79. [NEW] The method according to claim 78 wherein the one or more target nucleic acids are subjected to enzymatic cleavage reaction using one or more endonucleases, selected from the group consisting of restriction enzymes, RNA endonucleases, DNA endonucleases and non-specific phosphodiesterases.

80. [NEW] The method according to claim 79 wherein the one or more endonucleases are one or more selective or non-selective RNA endonucleases, selected from the group consisting of the G-specific T1 ribonuclease, the A-specific U2 ribonuclease, the A/U specific phyM ribonuclease, the U/C specific ribonuclease A, the C-specific chicken liver ribonuclease (RNaseCL3) and cusativin, non-specific RNase-I, and pyrimidine-adenosine preferring RNases isolated from E. coli, Enterobacter sp., or Saccharomyces cerevisiae.

81. [NEW] The method according to claim 57 wherein the one or more target nucleic acids are phosphorothioate-modified single stranded DNA or RNA, and wherein the cleavage reactions are performed with the nuclease P1.

82. [NEW] The method according to claim 57 wherein the one or more target nucleic acids are mosaic RNA/DNA nucleic acids or modified mosaic RNA/DNA nucleic acids, prepared with mutant polymerases, and wherein the cleavage reagents are RNA endonucleases, DNA endonucleases or alkali.

83. [NEW] The method according to claim 57 wherein the one or more target nucleic

acids are transcripts, modified transcripts, mosaic RNA/DNA transcripts or modified mosaic RNA/DNA transcripts, prepared with wild type or mutant RNA polymerases, and wherein the cleavage reagents are one or more selective or non-selective RNA endonucleases or alkali.

84. [NEW] The method according to claim 57 wherein the one or more target nucleic acids are mosaic RNA/DNA transcripts that incorporate either dCMP, dUMP or dTMP, prepared with mutant T7 or SP6 polymerase, and wherein the cleavage reagent is a pyrimidine-specific RNase, such as RNase-A.

85. [NEW] The method according to claim 57 wherein the set of non-ordered fragments of step (b) is additionally purified using an ion exchange beads.

86. [NEW] The method according to claim 57 wherein the set of non-ordered fragments of step (b) is spotted onto a solid support.

87. [NEW] The method according to claim 86 wherein said solid support is chosen from a group consisting of solid surfaces, plates and chips.

88. [NEW] The method according to claim 57 wherein the mass spectrometric analysis of the nucleic acid fragments is performed using a mass spectrometric method selected from the group consisting of Matrix-Assisted Laser Desorption/Ionization-Time-of-flight (MALDI-TOF), Electrospray-Ionization (ESI), and Fourier Transform-Ion Cyclotron Resonance (FT-ICR).

89. [NEW] The method according to claim 57 for sequence analysis of one or more target nucleic acids for which a reference nucleic acid sequence is known; comprising an additional step wherein the one or more mass spectra of the non-ordered fragments obtained in step c) are compared with the known or predicted mass spectra for a reference nucleic acid sequence, and deducing therefrom, by systematic computational analysis, all or part of the nucleotide sequence of the one or more target nucleic acids, and comparing the deduced nucleic acid sequence with the reference nucleic acid to determine whether the one or more

target nucleic acids have the same sequence or a different sequence from the reference nucleic acid.

90. [NEW] The method according to claim 89 wherein the nucleic acid sequence difference that is determined is a deletion, substitution, insertion or combinations thereof.

91. [NEW] The method according to claim 90 wherein the nucleic acid sequence difference is a Single Nucleic Polymorphism (SNP).

92. [NEW] Use of a method according to claim 89 for scoring known as well as unknown nucleotide sequence variations of one or more target nucleic acids present in one or more biological samples.

93. [NEW] Use according to claim 92 wherein the determination of said known or unknown nucleotide sequence variations allows the identification of the various allelic sequences of a certain region/gene, the scoring of disease-associated mutations, the detection of somatic variations, or studies in the field of molecular evolution.

94. [NEW] Use of a method according to claim 86 wherein the spectra obtained for one or more target nucleic acids are compared with the mass spectra predicted for a plurality of reference nucleic acids thereby identifying/detecting one or more target nucleic acids in one or more biological samples.

95. [NEW] Use according to claim 94 to determine the expression profile of one or more target nucleic acids in one or more biological samples.

96. [NEW] A method according to claim 57 for sequence analysis of one or more target nucleic acids of unknown sequence present in one or more biological samples, said method comprising the steps of:

(a)   deriving from one or more biological samples one or more target nucleic acids in a single stranded form;

(b)   subjecting the one or more target nucleic acids obtained from step (a) to a set of four separate base-specific complementary cleavage reactions, wherein each cleavage reaction generates a non-ordered set of fragments;

(c)   analyzing the sets of non-ordered fragments obtained from step (b) by mass spectrometry;

(d)   performing a systematic computational analysis on the mass spectra obtained from step (c) to assemble the sequence of said target nucleic acid; and,

(e)   optionally, if the sequence is not uniquely defined after step (d), repeating steps (a) through (d), thereby generating modified forms of said target nucleic acid and/or different portions of said target nucleic acid, and performing supplementary mono- and/or di-nucleotide specific cleavage reactions rendering supplementary sets of non-ordered fragments until the combined data converge into a unique sequence solution,

wherein said complementary cleavage reactions refer to target nucleic acid digestions characterized by varying specificity and/or to digestion of alternative forms of the target sequence.

97. [NEW] Use of a method according to claim 96 for the sequence analysis of one or more target nucleic acids of unknown sequence present in one or more biological samples.

98. [NEW] Use of a method according claim 96 for the sequence determination of one or more target nucleic acids of unknown sequence present in one or more biological samples.

99. [NEW] Use of a method according to claim 89 for genome wide genotyping of one or more biological samples.

100. A kit for sequence analysis according to a method of claim 57 of one or more target nucleic acids for which a reference nucleic acid sequence is known in one or more biological samples using mass spectrometry, the kit comprising:

(a)   one or more nucleotide triphosphates;

(b)   one or more polymerases;

(c)   one or more nucleic acid cleaving agents; and,

(d)   one or more sets of reference nucleic acids for which the nucleic acid sequence
is known;

(e)   optionally, reagents to purify the target nucleic acid;

(f)   optionally, ion exchange beads in order to purify the non-ordered set of .
fragments;

(g)   optionally, a solid support whereon the non-ordered set of fragments may be
spotted; and,

(h)   optionally, computer software for comparing the mass spectra of the one or
more target nucleic acid with the mass spectra of the reference nucleic acid
and deducing therefrom the nucleic acid sequence of the target nucleic acid.

101. [NEW] Use of a kit according to claim 100 for analyzing the sequence of, for
determining sequence differences in, for scoring known as well as unknown nucleotide
sequence variations in, for detecting/identifying, or, for performing genome wide genotyping
using one or more target nucleic acids, for which a reference nucleic acid sequence is known,
present in one or more biological samples.

102. [NEW] A kit for sequence analysis according to a method of claim 57 of one or
more unknown target nucleic acids in one or more biological sample using mass
spectroscopy, the kit comprising:

(a)   one or more nucleotide triphosphates;

(b)   one or more polymerases; and,

(c)   one or more nucleic acid cleaving agents;

(d)   optionally, reagents to purify the target nucleic acid;

(e)   optionally, ion exchange beads in order to purify the non-ordered set of
fragments;

(f)   optionally, a solid support whereon the non-ordered set of fragments may be
spotted; and,

(g)   optionally, computer software for analysing the sequence of said target nucleic

acid resulting in one or more unique sequences.

103. [NEW] Use of a kit according to claim 102 for analyzing the sequence of, for determining the sequence of, or, for performing genome wide genotyping using one or more target nucleic acids of unknown sequence present in one or more biological samples.

REMARKS

Applicants request that the above amendment be entered in the instant application prior to substantive examination.

Applicants have cancelled originally filed claims 1-56 and replaced them with new claims 57-103. Many of the new claims contain language similar to that of the claims as initially filed but in order to avoid any possible confusion that may be caused through the entry of amendments, Applicants have rewritten the claims. All of the new claims are fully supported by the specification as filed. For example, attached herewith as Appendix A is a table showing either the original claim and/or the page and line numbers in the specification where support for the new claims may be found. This amendment does not add new matter to the specification.

In light of the above amendments and additions, the claims presented for examination in the instant application are claims 57-103 (a clean copy of these claims is attached as Appendix B, for the Examiner's convenience). Applicants believe the presented claims are in condition for allowance and respectfully request an early indication of such favorable outcome.

Should the Examiner have any questions regarding this submission or any other matter relating to this application, Applicants invite the Examiner to telephone the undersigned representative.

Respectfully submitted,

MARSHALL, GERSTEIN & BORUN

February 7, 2002   By: _____

Nabeela R. McMillian
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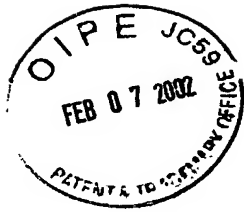
10018453.103001

APPENDIX A
SUPPORT FOR NEW CLAIMS

CLAIM NUMBER	SUPPORT: SPECIFICATION PAGE AND LINE NUMBER
57	original claim 1
58	original claim 3
59	original claim 4
60	original claim 5
61	original claim 6
62	page 22, lines 22-23
63	original claim 7
64	original claim 8
65	original 9 and page 24 lines 3-17
66	original claims 8 and 9
67	original claim 10
68	original claim 11
69	page 24 lines 3-17
70	original claim 13
71	original claim 14
72	page 29 lines 16-17
73	page 43 lines 14-25
74	page 43 lines 14-25
75	original claims 17 and 42
76	original claim 18
77	original claim 19 and page 29 lines 19-21
78	original claim 20
79	original claim 21
80	original claims 22 and 23
81	original claim 24

82	original claims 19 and 21 and page 7 lines 27-30
83	original claim 22
84	page 23 lines 25-30 and Example 2 at pages 41-44
85	page 42 lines 16-20
86	page 3 line 2; page 42 line 21 and page 43 line 26
87	page 3 line 2; page 42 line 21 and page 43 line 26
88	original claim 25
89	original claim 51
90	original claim 27
91	page 5 line 17
92	original claim 53
93	page 1 lines 5-16 and page 7 lines 2-12
94	original claim 53
95	original claim 55
96	original claims 1, 51 and 53; page 17 lines 4-10; and page 18 line 4 -page 21 line 25
97	original claim 53
98	original claim 51
99	original claim 52
100	original claim 56
101	original claim 56
102	original claim 56
103	original claim 56; page 3 line 2, page 42 lines 16-22 and page 43 line 26;

57. [NEW] A method for sequence analysis of one or more target nucleic acids present in one or more biological samples, said method comprising the steps of:

(a) deriving from one or more biological samples the one or more target nucleic acids;

(b) subjecting the one or more target nucleic acids obtained from step (a) to a set of separate base-specific, sequence-specific or site-specific complementary cleavage reactions, wherein each cleavage reaction generates a non-ordered set of fragments;

(c) analyzing the sets of non-ordered fragments obtained from step (b) by mass spectrometry; and,

(d) performing a systematic computational analysis on the mass spectra obtained from step (c) to analyse the sequence of said target nucleic acid,

wherein said complementary cleavage reactions refer to target nucleic acid digestions characterized by varying specificity and/or to digestion of alternative forms of the target sequence.

58. [NEW] The method according to claim 57 wherein the one or more biological samples are derived from organism selected from the group consisting of eukaryotes, prokaryotes, and viruses.

59. [NEW] The method according to claim 57 wherein the one or more target nucleic acids are selected from the group consisting of single stranded DNA, double stranded DNA, cDNA, single stranded RNA, double stranded RNA, DNA/RNA hybrid, and DNA/RNA mosaic nucleic acid.

60. [NEW] The method according to claim 57 wherein one or more target nucleic acids are derived by one or more consecutive amplification procedures selected from the group consisting of in vivo cloning, polymerase chain reaction (PCR), reverse transcription

followed by the polymerase chain reaction (RT-PCR), strand displacement amplification (SDA), and transcription based processes.

61. [NEW] The method according to claim 4 wherein the one or more amplified target nucleic acids are transcripts generated from a single stranded or a double stranded target nucleic acid by a process comprising the steps of:

(a)   linking operatively a transcription control sequences to the one or more target nucleic acids; and

(b)   transcribing one or both strands of the one or more target nucleic acid of step a) using one or more RNA polymerases that recognize the transcription control sequence on the one or more target nucleic acids.

62. [NEW] The method according to claim 61 wherein said transcriptional control sequences are operatively linked to one or more target nucleic acids by PCR amplification using primers that incorporate the transcriptional control sequences as 5'-extensions.

63. [NEW] The method according to claim 61 wherein the transcription control sequence is selected from the group consisting of eukaryotic transcription control sequences, prokaryotic transcription control sequences, and viral transcription control sequences.

64. [NEW] The method according to claim 63 wherein the prokaryotic transcription control sequence is selected from the group consisting of T3, T7, and SP6 promoters.

65. [NEW] The method according to claim 64 wherein the RNA polymerases which utilize the T3, T7, or SP6 promoters are either wild type or mutant RNA polymerases, the mutant polymerases being capable of incorporating into the transcript non-canonical substrates with a 2'-deoxy, 2'-O-methyl, 2'-fluoro or 2'-amino substituent.

66. [NEW] The method according to claim 65 wherein the mutant RNA polymerase is either T7 or SP6 mutant polymerase.

67. [NEW] The method according to claim 57 wherein the derived target nucleic acid incorporates one or more nucleosides that are modified on the base, the sugar, and/or the phosphate moiety, wherein the modifications alter the specificity of cleavage by the one or more cleavage reagents and/or the mass and/or the length of the cleavage products.

68. [NEW] The method according to claim 67 wherein the modification is introduced through the enzymatic incorporation of modified deoxynucleoside triphosphates, modified ribonucleoside triphosphates, and/or modified dideoxynucleoside triphosphates; or wherein the modification is introduced chemically, or wherein the modification is introduced through a combination of both methods.

69. [NEW] The method according to claim 67 wherein the modification consists of a 2'-deoxy, 2'-O-methyl, 2'-fluoro or 2'-amino substituent on the nucleotide triphosphates.

70. [NEW] The method according to claim 67 wherein the modification consists of phosphorothioate internucleoside linkages or phosphorothioate internucleoside linkages further reacted with an alkylating reagent.

71. [NEW] The method according to claim 67 wherein the modification consists of a methyl group on C5 of the uridine-5'-monophosphate subunits.

72. [NEW] The method according to claim 67 wherein the modification consists of nucleotides that incorporate alternative isotopes.

73. [NEW] The method according to claim 57 wherein the one or more target nucleic acids of step (a) are purified prior to cleavage.

74. [NEW] The method according to claim 73 wherein said purification is achieved through immobilization or by chromatography.

75. [NEW] The method according to claim 57 wherein the complementary cleavage

reactions are selected from the group consisting of enzymatic cleavage, chemical cleavage, and physical cleavage.

76. [NEW] The method according to claim 75 wherein the complementary cleavage reactions are characterized by a relaxed mono-nucleotide, mono-nucleotide, relaxed di-nucleotide, or di-nucleotide specificity.

77. [NEW] The method according to claim 75 wherein the one or more target nucleic acids are subjected to chemical digestion reaction consisting of treatment with alkali or with reagents used in the Maxam & Gilbert sequencing method.

78. [NEW] The method according to claim 75 wherein the one or more target nucleic acids are subjected to enzymatic cleavage reaction using one or more enzymes selected from the group consisting of endonucleases and exonucleases.

79. [NEW] The method according to claim 78 wherein the one or more target nucleic acids are subjected to enzymatic cleavage reaction using one or more endonucleases, selected from the group consisting of restriction enzymes, RNA endonucleases, DNA endonucleases and non-specific phosphodiesterases.

80. [NEW] The method according to claim 79 wherein the one or more endonucleases are one or more selective or non-selective RNA endonucleases, selected from the group consisting of the G-specific T1 ribonuclease, the A-specific U2 ribonuclease, the A/U specific phyM ribonuclease, the U/C specific ribonuclease A, the C-specific chicken liver ribonuclease (RNaseCL3) and cusativin, non-specific RNase-I, and pyrimidine-adenosine preferring RNases isolated from E. coli, Enterobacter sp., or Saccharomyces cerevisiae.

81. [NEW] The method according to claim 57 wherein the one or more target nucleic acids are phosphorothioate-modified single stranded DNA or RNA, and wherein the cleavage reactions are performed with the nuclease P1.

82. [NEW] The method according to claim 57 wherein the one or more target nucleic acids are mosaic RNA/DNA nucleic acids or modified mosaic RNA/DNA nucleic acids, prepared with mutant polymerases, and wherein the cleavage reagents are RNA endonucleases, DNA endonucleases or alkali.

83. [NEW] The method according to claim 57 wherein the one or more target nucleic acids are transcripts, modified transcripts, mosaic RNA/DNA transcripts or modified mosaic RNA/DNA transcripts, prepared with wild type or mutant RNA polymerases, and wherein the cleavage reagents are one or more selective or non-selective RNA endonucleases or alkali.

84. [NEW] The method according to claim 57 wherein the one or more target nucleic acids are mosaic RNA/DNA transcripts that incorporate either dCMP, dUMP or dTMP, prepared with mutant T7 or SP6 polymerase, and wherein the cleavage reagent is a pyrimidine-specific RNase, such as RNase-A.

85. [NEW] The method according to claim 57 wherein the set of non-ordered fragments of step (b) is additionally purified using an ion exchange beads.

86. [NEW] The method according to claim 57 wherein the set of non-ordered fragments of step (b) is spotted onto a solid support.

87. [NEW] The method according to claim 86 wherein said solid support is chosen from a group consisting of solid surfaces, plates and chips.

88. [NEW] The method according to claim 57 wherein the mass spectrometric analysis of the nucleic acid fragments is performed using a mass spectrometric method selected from the group consisting of Matrix-Assisted Laser Desorption/Ionization-Time-of-flight (MALDI-TOF), Electrospray-Ionization (ESI), and Fourier Transform-Ion Cyclotron Resonance (FT-ICR).

89. [NEW] The method according to claim 57 for sequence analysis of one or more

target nucleic acids for which a reference nucleic acid sequence is known; comprising an additional step wherein the one or more mass spectra of the non-ordered fragments obtained in step c) are compared with the known or predicted mass spectra for a reference nucleic acid sequence, and deducing therefrom, by systematic computational analysis, all or part of the nucleotide sequence of the one or more target nucleic acids, and comparing the deduced nucleic acid sequence with the reference nucleic acid to determine whether the one or more target nucleic acids have the same sequence or a different sequence from the reference nucleic acid.

90. [NEW] The method according to claim 89 wherein the nucleic acid sequence difference that is determined is a deletion, substitution, insertion or combinations thereof.

91. [NEW] The method according to claim 90 wherein the nucleic acid sequence difference is a Single Nucleic Polymorphism (SNP).

92. [NEW] Use of a method according to claim 89 for scoring known as well as unknown nucleotide sequence variations of one or more target nucleic acids present in one or more biological samples.

93. [NEW] Use according to claim 92 wherein the determination of said known or unknown nucleotide sequence variations allows the identification of the various allelic sequences of a certain region/gene, the scoring of disease-associated mutations, the detection of somatic variations, or studies in the field of molecular evolution.

94. [NEW] Use of a method according to claim 86 wherein the spectra obtained for one or more target nucleic acids are compared with the mass spectra predicted for a plurality of reference nucleic acids thereby identifying/detecting one or more target nucleic acids in one or more biological samples.

95. [NEW] Use according to claim 94 to determine the expression profile of one or more target nucleic acids in one or more biological samples.

96. [NEW] A method according to claim 57 for sequence analysis of one or more target nucleic acids of unknown sequence present in one or more biological samples, said method comprising the steps of:

(a)   deriving from one or more biological samples one or more target nucleic acids in a single stranded form;

(b)   subjecting the one or more target nucleic acids obtained from step (a) to a set of four separate base-specific complementary cleavage reactions, wherein each cleavage reaction generates a non-ordered set of fragments;

(c)   analyzing the sets of non-ordered fragments obtained from step (b) by mass spectrometry;

(d)   performing a systematic computational analysis on the mass spectra obtained from step (c) to assemble the sequence of said target nucleic acid; and,

(e)   optionally, if the sequence is not uniquely defined after step (d), repeating steps (a) through (d), thereby generating modified forms of said target nucleic acid and/or different portions of said target nucleic acid, and performing supplementary mono- and/or di-nucleotide specific cleavage reactions rendering supplementary sets of non-ordered fragments until the combined data converge into a unique sequence solution,

wherein said complementary cleavage reactions refer to target nucleic acid digestions characterized by varying specificity and/or to digestion of alternative forms of the target sequence.

97. [NEW] Use of a method according to claim 96 for the sequence analysis of one or more target nucleic acids of unknown sequence present in one or more biological samples.

98. [NEW] Use of a method according claim 96 for the sequence determination of one or more target nucleic acids of unknown sequence present in one or more biological samples.

99. [NEW] Use of a method according to claim 89 for genome wide genotyping of one or more biological samples.

100. A kit for sequence analysis according to a method of claim 57 of one or more target nucleic acids for which a reference nucleic acid sequence is known in one or more biological samples using mass spectrometry, the kit comprising·

- (a) one or more nucleotide triphosphates;
- (b) one or more polymerases;
- (c) one or more nucleic acid cleaving agents; and,
- (d) one or more sets of reference nucleic acids for which the nucleic acid sequence is known;
- (e) optionally, reagents to purify the target nucleic acid;
- (f) optionally, ion exchange beads in order to purify the non-ordered set of fragments;
- (g) optionally, a solid support whereon the non-ordered set of fragments may be spotted; and,
- (h) optionally, computer software for comparing the mass spectra of the one or more target nucleic acid with the mass spectra of the reference nucleic acid and deducing therefrom the nucleic acid sequence of the target nucleic acid.

101. [NEW] Use of a kit according to claim 100 for analyzing the sequence of, for determining sequence differences in, for scoring known as well as unknown nucleotide sequence variations in, for detecting/identifying, or, for performing genome wide genotyping using one or more target nucleic acids, for which a reference nucleic acid sequence is known, present in one or more biological samples.

102. [NEW] A kit for sequence analysis according to a method of claim 57 of one or more unknown target nucleic acids in one or more biological sample using mass spectroscopy, the kit comprising:

- (a) one or more nucleotide triphosphates;
- (b) one or more polymerases; and,
- (c) one or more nucleic acid cleaving agents;
- (d) optionally, reagents to purify the target nucleic acid;
- (e) optionally, ion exchange beads in order to purify the non-ordered set of

fragments;

(f)   optionally, a solid support whereon the non-ordered set of fragments may be spotted; and,

(g)   optionally, computer software for analysing the sequence of said target nucleic acid resulting in one or more unique sequences.

103. [NEW] Use of a kit according to claim 102 for analyzing the sequence of, for determining the sequence of, or, for performing genome wide genotyping using one or more target nucleic acids of unknown sequence present in one or more biological samples.

DIAGNOSTIC SEQUENCING BY A COMBINATION
OF SPECIFIC CLEAVAGE AND MASS SPECTROMETRY

FIELD OF INVENTION

5   The present invention is in the field of nucleic acid-based diagnostic

assays. More particularly, it relates to methods useful for the "diagnostic sequencing"

of regions of sample nucleic acids for which a prototypic or reference sequence is

already available (also referred to as 're-sequencing'), or which may be determined

using the methods described herein. This diagnostic technology is useful in areas that

10   require such re-sequencing in a rapid and reliable way: (i) the identification of the

various allelic sequences of a certain region/gene, (ii) the scoring of disease-associated

mutations, (iii) the detection of somatic variations, (iv) studies in the field of

molecular evolution, (v) the determination of the nucleic acid sequences of

prokaryotic and eukaryotic genomes; (vi) identifying one or more nucleic acids in one

15   or more biological samples; (vii) and determining the expression profile of genes in a

biological sample and other areas.

BACKGROUND OF INVENTION

         Complete reference genome sequences for a number of model

20   organisms as well as humans are currently available or are expected to become

available in the near future. A parallel challenge is to characterize the type and extent

of variation in the sequences of interest because it underlies the heritable differences

among individuals and populations. In humans, the vast majority of sequence

variation consists of nucleotide substitutions referred to as single nucleotide

25   polymorphisms (SNPs). DNA sequencing is the most sensitive method to discover

polymorphisms [Eng C. and Vijg J. *et al., Nature Biotechnol.* 15: 422-426 (1997)]. A

growing panel of such sequence variants, together with powerful methods to monitor

them [Landegren U. *et al., Genome Res.* 8: 769-776 (1998)], is useful in linkage

studies to identify even the most subtle disease susceptibility loci [Lander E. and

30   Schork N., *Science* 265: 2037-2048 (1994); Risch N. and Merikangas K., *Science* 273:

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1516-1517 (1996)]. Also, the identification of all (functional) allelic variants will require the re-sequencing of particular regions in a large number of samples [Nickerson D. *et al., Nature Genet.* 19: 233-240 (1998)]. Although a number of methods to monitor known SNPs have been developed [Landegren U. *et al., Genome*

5   *Res.* 8: 769-776 (1998)], re-sequencing is likely to be routinely applied to secure diagnoses of patients. Indeed, in a significant number of disease-associated genes that have been surveyed thus far, literally hundreds or even thousands of different mutations have been identified and catalogued. Consequently, sequence determination represents the ultimate level of resolution and may be the preferred method to monitor

10   which mutation or combination of mutations, out of a large number of mutations of known clinical relevance, is present.

It would appear that the field of biomedical genetics will rely heavily on sequencing technology. Hence, there is a need for advanced sequencing methods that are time- and cost-competitive, and at the same time accurate and robust. Recent

15   developments in this area include improvements to the basic dideoxy chain termination sequencing method [Sanger *et al. Proc. Natl. Acad. Sci. USA* 74: 5463-5467 (1977); reviewed by Lipshutz R. and Fodor S. *et al., Current Opinion in Structural Biology* 4: 376-380 (1994)], as well as new approaches that are based on entirely new paradigms. Two such novel approaches are sequencing-by-hybridization

20   (SBH) [Drmanac R. *et al., Science* 260: 1649-1652 (1993)] and pyro-sequencing [Ronaghi M. *et al., Science* 281: 363-365 (1998); Ronaghi M. *et al., Anal. Biochem.* 242: 84-89 (1996)]. While the concepts of these approaches have been experimentally validated, their ultimate acceptance and usage may depend on the type of application — *e.g. de novo* sequencing, re-sequencing, and genotyping of known SNPs.

25   Recently, progress has also been made in the use of mass spectroscopy (MS) to analyze nucleic acids [Crain, P.F. and McCloskey, J.A., *Current Opinion in Biotechnology* 9: 25-34 (1998), and references cited therein]. One promising development has been the application of MS to the sequence determination of DNA and RNA oligonucleotides [Limbach P., *Mass Spectrom. Rev. 15*: 297-336 (1996);

30   Murray K., *J. Mass Spectrom.* 31: 1203-1215 (1996)]. MS and more particularly,

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matrix-assisted laser desorption/ionization MS (MALDI MS) has the potential of very

high throughput due to high-speed signal acquisition and automated analysis off solid

surfaces. It has been pointed out that MS, in addition to saving time, measures an

intrinsic property of the molecules, and therefore yields a significantly more

5   informative signal [Köster H. *et al.*, *Nature Biotechnol.*, **14**: 1123-1128 (1996)].

Sequence information can be derived directly from gas-phase

fragmentation [see for example Nordhoff E. *et al.*, *J. Mass Spectrom.*, **30**:99-112

(1995); Little D. *et al.*, *J. A. Chem. Soc.*, **116**: 4893-4897 (1994); Wang B. *et al.*, WO

98/03684 and WO 98/40520; Blöcker H. *et al.*, EP 0 103 677; Foote S. *et al.*, WO

10   98/54571]. In contrast, indirect methods measure the mass of fragments obtained by a

variety of methods in the solution phase, *i.e.*, prior to the generation of gas phase ions.

In its simplest form, mass analysis replaces the gel-electrophoretic fractionation of

the fragment-ladder (*i.e.*, a nested set of fragments that share one common endpoint)

generated by the sequencing reactions. The sequencing reactions need not necessarily

15   be base-specific because the base-calling may also be based on accurate mass

measurement of fragments that terminate at successive positions and that differ from

one another by one nucleotide residue. The fragment-ladder can be generated by the

Sanger method [Köster H. *et al.*, *Nature Biotechnol.*, **14**: 1123-1128 (1996); Reeve

M.A., Howe R. P., Schwarz T., U.S. 5,849,542; Köster H., U.S. 5,547,835; Levis R.

20   and Romano L., U.S. 5,210,412 and U.S. 5,580,733; Chait B. and Beavis R., U.S.

5,453,247], by base-specific partial RNA digestion [Hahner S. *et al.*, *Nucleic Acids

Res.*, **25**: 1957-1964 (1997); Köster H., WO 98/20166] or by chemical cleavage [Isola

N. *et al.*, *Anal. Chem.*, **71**: 2266-2269 (1999); references cited in Limbach P., *Mass

Spectrom. Rev.*, **15**: 297-336 (1996)]. An alternative method consists of analyzing

25   the ladder generated by exonuclease digestion from either the 3'- or 5'-end [Pieles U.

et al., *Nucleic Acids Res.*, **21**: 3191-3196 (1993); Köster H., U.S. 5,851,765; Engels J.

et al., WO 98/45700; Tarr G. and Patterson D., WO 96/36986; Patterson D., U.S.

5,869,240].

A severe limitation of both the direct and indirect MS methodologies

30   under the current performance conditions is the poor applicability to chain lengths

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beyond ~30-50 nucleotides. As a consequence, it has been suggested that the

prospects for MS lie with DNA diagnostic assays, rather than large-scale sequencing

[Smith L., *Nature Biotechnol.*, 14: 1084-1087 (1996)]. Given the fact that MS

represents an exquisite means to analyze short nucleotide fragments, the various MS-

5   based processes that have been described for nucleic acid based diagnostic purposes

generally involve the derivation and analysis of such relatively short fragments [see

for example Köster H., WO 96/29431; Köster H. *et al.*, WO 98/20166; Shaler T. *et*

al., WO 98/12355; Kamb A., U.S. 5,869,242; Monforte J. *et al.*, WO 97/33000; Foote

S. *et al.*, WO 98/54571].

10   Some of the MS-based assays have been used for the scoring of

defined mutations or polymorphisms. Other processes derive multiple

oligonucleotide fragments and yield a 'mass-fingerprint' so as to analyze a larger

target nucleic acid region for mutations and/or polymorphisms. The latter MS

analyses are however considerably less informative in that they are essentially

15   restricted to the detection of sequence variations. The methods cannot be applied to

diagnostic sequencing of nucleic acids, where the term diagnostic sequencing means

the unequivocal determination of the presence, the nature and the position of sequence

variations. At best, the measurements confirm the base composition of small

fragments whose masses are determined with sufficient accuracy to reduce the number

20   of possible compositional isomers. Also, it will be realized that only certain changes

in composition (as revealed by shifts in the mass spectrum) can be unambiguously

assigned to a polymorphism or mutation. A match between the spectrum of the

interrogated sequence and a reference-spectrum obtained from wild-type sequence or

sequences known to contain a given polymorphism, is assumed to indicate that the

25   interrogated nucleic acid region is wild-type or incorporates the previously known

polymorphisms, thereby disregarding certain other possible interpretations.

While most methods in the art do yield sequence-related information,

they do not disclose that a combination of several different mass spectra, obtained

after complementary digestion reactions, allows for the effective survey of a nucleic

30   acid region and provides an unambiguous assignment of both known as well as

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previously unknown sequence variations that occur relative to a reference nucleic acid with a known nucleotide sequence.

In view of the limitations of the methods described above, the art would clearly benefit from a new procedure for the diagnostic sequencing of nucleic
5   acids that would overcome the shortcomings of the processes discussed above.

In comparison with conventional sequencing technology, *i.e.*, the gel-electrophoretic analysis of fragment ladders, the methods of the present invention are more suited for the simultaneous analysis of multiple target sequences. In general, each particular sequence or sequence variant is associated with a distinct set of mass
10   peaks. Consequently, the sequencing reactions according to the methods of the present invention lend themselves readily to (i) multiplexing (*i.e.*, the analysis of two or more target non-contiguous target regions from a single biological sample), (ii) the analysis of heterozygous samples, as well as (iii) pooling strategies (*i.e.*, the simultaneous sequencing of the analogous regions derived from two or more different
15   biological samples).

Because of the multiplex capacity, the present methods can be adapted as a tool for the genome-wide discovery and scoring of polymorphisms (*e.g.*, SNPs) useful as markers in genetic linkage studies. The unambiguous identification/diagnosing of a number of variant positions is less demanding than full
20   sequencing and, consequently, a considerable number of target genomic loci can be combined and analyzed at the same time, especially when their lengths are kept relatively small. The number of markers that can be scored in parallel will depend on the level of genetic diversity in the species of interest and on the precise method used to prepare and analyze the target nucleic acids, but may typically be in the order of a
25   few tens to up to 100 with current MS capabilities. The addition of multiplexing to the high-precision and high-speed characteristics of MS constitutes a new marker technology that enables the large-scale and cost-effective scoring of several (tens of) thousands of markers. Some aspects of the application of the present methods to genome-wide genotyping are described in Example 5.

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Sequencing reactions according to the methods of the present invention yield, in principle, a discrete set of fragments for each individual sequence or sequence variant whereas conventional sequence ladders stack on top of one another. Therefore, such sequences or sequence variants can be analyzed even when present as

5   a lesser species. This is a useful quality for the analysis of clinical samples which are often genetically heterogeneous because of the presence of both normal and diseased cells or in itself (*e.g.*, cancerous tissue, viral quasi-species). Additionally, the ability to detect mutations at a low ratio of mutant over wild-type allele makes it practicable to pool individual biological samples, a strategy which should permit a more cost-

10   effective search for genomic sequence variations in a population.

The present invention rests in part on the insight that integration of the data obtained in a set of complementary fingerprints produced by an appropriate set of complementary cleavage reactions of the invention represents a level of characterization of a sample nucleic acid essentially equal to sequence determination.

15   The present invention is also directed to the use of cleavage protocols that result in the generation of cleavage products that range from mono- and dinucleotides to fragments of a few tens of nucleotides that are particularly suited for analysis by MS. At the same time, the present method is distinct from the other fragmentation processes that are limited to screening target nucleic acids for a wide range of potential mutations.

20   According to the present invention, a combination of several different mass spectra, obtained after complementary digestion reactions, coupled with systematic computational analysis allows the survey of a selected target nucleic acid or region thereof and leads to the unambiguous assignment of both known and previously unknown sequence variations. In certain aspects of the present invention, knowledge

25   of the reference sequence in combination with the methods disclosed herein allows modeling of the experimental approach, anticipation of potential ambiguities, and the design of an adequate resolution.

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SUMMARY OF INVENTION

The present invention is directed to a mass spectroscopic method for detecting or analyzing a particular nucleic acid sequence. The present invention is useful for *de novo* sequencing or re-sequencing nucleic acid in a rapid and reliable

5   way which permits, for example, the identification of the various allelic sequences of a certain region/gene, the identification and scoring of disease-associated mutations, the detection of somatic variations, determining genetic diversity in molecular evolution, and the determination of the genomic sequences *e.g.*, of viral and bacterial isolates. The present invention is also useful for identification of all nucleic acid

10   molecules in one or more biological samples including for expression profiling *i.e.*, identification of all the mRNA species that are expressed in a given cell at a given time, by rapidly determining the sequence of the mRNA that is expressed.

In one embodiment, the present invention is directed to methods for sequence analysis of one or more target nucleic acids for which a known reference

15   nucleic acid sequence is available. In this method, one or more target nucleic acids are derived from one or more biological samples, and a reference nucleic acid are each subjected to complementary cleavage reactions, and the products of the cleavage reactions are analyzed by mass spectroscopic methods. The mass spectra of the one or more target nucleic acids are then compared with the mass spectra of the reference

20   nucleic acid sequence, and the nucleotide sequence of the one or more target nucleic acids is deduced by systematic computational analysis.

In one aspect of this embodiment, multiple targets, such as cDNA clones, are prepared from the mRNA of the same biological sample, and are separately analyzed as above in parallel experiments. In a second aspect, multiple

25   targets are derived from the same biological sample and are analyzed simultaneously, for example in genome-wide genotyping.

The one or more target nucleic acids may be selected from the group consisting of a single stranded DNA, a double stranded DNA, a cDNA, a single stranded RNA, a double stranded RNA, a DNA/RNA hybrid, and a DNA/RNA

30   mosaic nucleic acid.

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In a second embodiment, the one or more target nucleic acids are selected from the group consisting of an amplified nucleic acid fragment, a cloned nucleic acid fragment, and a series of non-contiguous DNA fragments from the genome. In one aspect of this invention, the amplified one or more target nucleic

5   acids are derived by one or more consecutive amplification procedures selected from the group consisting of *in vivo* cloning, the polymerase chain reaction (PCR), reverse transcription followed by the polymerase chain reaction (RT-PCR), strand displacement amplification (SDA), and transcription based processes.

In a preferred embodiment, the amplified nucleic acid fragments are

10   RNA transcripts generated from one or more target nucleic acids or a reference nucleic acid by a process comprising the steps of: (a) amplifying the one or more target nucleic acids or the reference nucleic acid using one or more primers corresponding to a region that is complementary to the one or more target nucleic acids or the reference nucleic acid and encoding an expression control sequence using

15   any one of the amplification procedures described above, and (b) generating RNA transcripts from the amplified one or more target nucleic acids or reference nucleic acid using one or more RNA polymerases that recognize the transcription control sequence on the target or reference nucleic acid. The RNA generated by the above process is then subjected to complementary cleavage reactions to generate nucleic

20   acid fragments, which are then analyzed by MS. The transcription control sequence may be selected from the group consisting of an eukaryotic transcription control sequence, a prokaryotic transcription control sequence, and a viral transcription control sequence. The prokaryotic transcription control sequence may be selected from the group consisting of T3, T7, and SP6 promoters. The cognate RNA

25   polymerases may be either a wild-type or a mutant form capable of incorporating non-canonical substrates with a 2'-substituent other than a hydroxyl group.

In a third embodiment, the one or more target nucleic acids are amplified using modified nucleoside triphosphates. The mass modified nucleoside triphosphates may be selected from the group consisting of a mass modified

30   deoxynucleoside triphosphate, a mass modified dideoxynucleoside triphosphate, and a

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mass modified ribonucleoside triphosphate. The mass modified nucleoside triphosphate may be modified on the base, the sugar, and/or the phosphate moiety, and are introduced through an enzymatic step, chemically, or a combination of both. In one aspect the modification may consist of 2'-substituents other than a hydroxyl group

5   on transcript subunits. In another aspect, the modification may consist of phosphorothioate internucleoside linkages or phosphorothioate internucleoside linkages further reacted with an alkylating reagent. In yet another aspect, the modification may consist of a methyl group on C5 of the uridine-5'-monophosphate subunits. Such modifications may alter the specificity of cleavage by certain reagents,

10   and/or the mass of the cleavage products, and/or the length of the cleavage products.

In one aspect of the invention, the one or more target nucleic acids and reference nucleic acid are subjected to complementary cleavage reactions using enzymatic cleavage, chemical cleavage, and/or physical cleavage reactions. In a preferred embodiment, the one or more target nucleic acids and the reference nucleic

15   acid are subjected to enzymatic cleavage reaction using one or more enzymes selected from the group consisting of endonucleases and exonucleases. In a more preferred embodiment, the target nucleic acid is a double-stranded RNA and the endonuclease used is a ribonuclease. The ribonuclease may be selected the G-specific T_1 ribonuclease, the A-specific U_2 ribonuclease, the A/U specific phyM ribonuclease, the

20   U/C specific ribonuclease A, the C-specific chicken liver ribonuclease (RNaseCL3), and cusativin. In one aspect of this preferred embodiment, the target nucleic acid is a phosphorothioate-modified single-stranded DNA or RNA and the endonuclease is nuclease P1.

In another aspect, the mass spectroscopical analysis of the nucleic acid

25   fragments is performed using a mass spectrometer selected from the group consisting of Matrix-Assisted Laser Desorption/Ionization-Time-of-flight (MALDI-TOF), Electrospray-Ionization (ESI), and Fourier Transform-Ion Cyclotron Resonance (FT-ICR). In a preferred embodiment the mass spectrometer used for the analysis of the cleavage fragments is MALDI-TOF

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In a fifth embodiment, the method of the present invention can be used for diagnosing nucleic acid sequence variations in one or more target nucleic acids derived from a biological sample, for which a known reference nucleic acid sequence is available. In this method, one or more target nucleic acids derived from a biological

5   sample, and a reference nucleic acid whose sequence has been predetermined are subjected to complementary cleavage reactions, and the products of the cleavage reactions are analyzed by mass spectroscopic methods. The mass spectra of the one or more target nucleic acids is then compared with the mass spectra of the reference nucleic acid, and the nucleotide sequence variations in the one or more target nucleic

10   acids is then deduced by systematic computational analysis of the sequence variations between the one or more target nucleic acids and the reference nucleic acid. A variety of acid sequence variations including deletions, substitutions and/or insertions in a target nucleic acid can be determined using the method of the present invention.

In a sixth embodiment, the method of the present invention can be used

15   for scoring known nucleotide sequence variations in one or more target nucleic acids derived from a biological sample, for which a known reference nucleic acid sequence is available. In this embodiment, one or more target nucleic acids derived from a biological sample, and a reference nucleic acid are subjected to complementary cleavage reactions, and the products of the cleavage reactions are analyzed by mass

20   spectroscopic methods. The mass spectra of the one or more target nucleic acid is then compared with the mass spectra of the reference nucleic acid sequence, and the nucleotide sequence variations/mutations in the one or more target nucleic acids are scored by comparing the nucleic sequence between the one or more target nucleic acid and reference nucleic acid by systematic computational analysis.

25   In a seventh embodiment, the method of the present invention can be used for determining the nucleotide sequence (*de novo sequencing*) of one or more target nucleic acids derived from a biological sample for which no reference sequence is available. In this method, target nucleic acid, derived from a biological sample is subjected to complementary cleavage reactions, and the products of the cleavage

30   reactions are analyzed by mass spectroscopic methods. The mass spectra of the one

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or more target nucleic acids coupled with a systematic computational analysis is then used to deduce the sequence of the one or more target nucleic acids.

In an eighth embodiment, the method of the present invention can be used for genome-wide genotyping of one or more known or unknown target nucleic

5   acids. In this method, one or more target nucleic acids, derived from a biological sample, are amplified and then subjected to complementary cleavage reactions. In one aspect, multiple targets are derived from a single sample and are analyzed simultaneously. The products of the cleavage reactions are then analyzed by mass spectroscopic methods. The mass spectra of the one or more known or unknown

10   target nucleic acid is compared with the mass spectra of a reference nucleic acid. This comparison is then used to infer the genotype of an organism from which the biological sample is derived and to determine therefrom the genetically relevant nucleic acid sequence variations of the one or more known or unknown nucleic acids.

In a ninth embodiment, the method of the present invention can be

15   used to identify one or more target nucleic acids in one or more biological samples. In this method, one or more target nucleic acids, derived from a biological sample, are amplified and then subjected to complementary cleavage reactions. In one aspect, multiple targets are derived from a single sample and are analyzed simultaneously. The products of the cleavage reactions are then analyzed by mass spectroscopic

20   methods. The identity of one or more target nucleic acids is deduced by comparing the mass spectra of the one or more known or unknown target nucleic acid with each other or by comparison with a plurality of mass spectra of reference nucleic acids.

In one aspect, the method of the present invention can be used for expression profiling, *i.e.* identifying the various mRNA expressed in one or more

25   biological samples.

Also encompassed by the present invention is a kit for sequence analysis of one or more target nucleic acids using mass spectroscopy, the kit comprising a container having one or more sets of reference nucleic acids for which the nucleotide sequence is known, one or more nucleic acid cleaving agents, and

30   computer algorithm/software for comparing the mass spectra of the one or more target

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nucleic acids with the mass spectra of the reference nucleic acid and deducing

therefrom the nucleic acid sequence of the one or more target nucleic acids. In one

embodiment, the nucleic acid cleaving agent in the kit is a chemical agent. In an

alternate embodiment, the nucleic acid cleaving agent is an enzyme selected from a

5   group of enzymes consisting of endonucleases and exonucleases. In a preferred

embodiment, the endonuclease is a ribonuclease selected from the group consisting of

the G-specific T_1 ribonuclease, the A-specific U_2 ribonuclease, the A/U specific phyM

ribonuclease, the U/C specific ribonuclease A, the C-specific chicken liver

ribonuclease (RNaseCL3), and cusativin.

10

DESCRIPTION OF DRAWINGS

Figure 1A (SEQ ID NO: 1) graphically represents the first 120

nucleotides of exon 5 of human p53 as well as the fragments that would result from

cleavage of the (+) and (-) strand transcript after G (RNase-T1) or A (RNase-U2). The

15   dotted and full arrows correspond to the resulting ≤3-mer and ≥4-mer cleavage

products. The arrows from left to right represent fragments from the (+) strand, while

the arrows from right to left represent fragments from the (-) strand. The numbers

indicate the neutral molecular masses of the ≥4-mer ribonucleotide fragments. The

calculation assumes that all fragments contain 5'-hydroxyl and 3'-phosphate groups.

20   Figure 1B shows the size distribution of the products that result from

base-specific cleavage of a 245 nucleotides long exemplary sequence.

Figure 2 summarizes the results of the mutational simulation analysis

of a 200-base-pair segment of the HIV protease gene and shows the percentages of the

mutational changes that can be detected (hatched bars) and mapped (filled bars). The

25   results were computed for single RNase digests of the (+) and (-) strands with

respectively RNase-T1 (T1) and RNase-U2 (U2), separately or combined (T1/U2). All

refers to the analysis with the four different reactions.

Figure 3 shows the distributions of the number of diagnostic fragments

obtained for the mutational simulation analysis of 1.200 base-pair sequence of HIV

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when using different length segments of respectively 100, 200, 300, and 600 base-pairs.

Figure 4 summarizes the results of the mutational simulation analysis of 1.200 base-pair sequence of HIV and shows the percentages of the single

5   nucleotide substitutions that can be detected (hatched bars) and mapped unambiguously (filled bars) as a function of the length of the interrogated segments.

Figure 5 (SEQ ID NO: 2 and SEQ ID NO: 3) is a graphic representation of the pGEM3-Zf(+) derived nucleotide sequences used as a model in Examples 2 and 4. The regions corresponding to the PCR primers are underlined. Two

10   PCR products (158 and 1012 base-pairs long) were generated. Both amplification products encompass the phage T7 promoter site; the transcription initiation site is indicated with an arrow. The numbering refers to the respective transcripts (118 and 972 nucleotides).

Figure 6 is a graphical representation of the MALDI-TOF mass spectra

15   of the RNase-A cleavage reactions of pGEM3-Zf(+) derived transcripts. The following transcripts were digested: (A) a regular transcript synthesized with rNTPs, (B) a transcript in which UMP residues are replaced by dTMP, (C) a transcript where UMP is replaced by dUMP, and (D) one that incorporates dCMP instead of CMP. Observed masses are indicated above the peaks that match with predicted digestion

20   products (see Table II).

Figure 7A (SEQ ID NO: 4 and SEQ ID NO: 5) is a graphical representation of PCR products and transcripts used for diagnostic sequencing of the RNase-T1 coding region. Two parallel amplification reactions were performed with either the upstream or downstream primer tagged to the T7 promoter. The

25   amplification products allow the transcription of the (+; upper sequence) or (-; lower sequence) strand. The underlined region shows the appended T7 promoter site. An arrow indicates the transcription initiation site.

Figure 7B (SEQ ID NO: 6 through SEQ ID NO: 14)shows the position and nature of a number of single, double, and triple mutations in RNase-T1 (reference

30   denotes the wild-type coding region).

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Figure 8 is a graphical representation of the MALDI-TOF mass spectra obtained for RNase-T1 analysis. Four transcripts were digested with RNase-A: (A) dU-incorporating transcript of the (+) strand, (B) dC-transcript of the (+) strand, (C) dU-transcript of the (-) strand, (D) dC-transcript of the (-) strand. The observed

5   masses of predicted peaks are indicated. Presumed double protonated peaks are labeled M^{2+} with the mass of the parental $[M+H]^+$ peak indicated between parentheses (Figure 8B). One of the peaks in Figure 8D (1207.1+G) is best explained by assuming the addition of an extra G-residue at the transcript 3'-end. Figure 8C only shows the 900-4800 Da mass range; the digestion product of 11124 Da was not detected.

10   Figure 9 (panels A, B, and C) is a graphical representation of the MALDI-TOF mass spectra of the RNase-A cleavage reaction of a pGEM3-Zf(+) derived T7-transcript of 972 nucleotides long. The transcript incorporates dCMP instead of CMP residues. The observed masses of the predicted peaks is indicated. An asterisk indicates 2',3'-cyclic phosphate reaction intermediates (see Table V).

15

DETAILED DESCRIPTION OF INVENTION

With current capabilities in mass spectroscopy, it is impractical to sequence nucleic acids greater than ~50 bases in length. Consequently, an impractical and cumbersome number of independent sequencing reactions is necessary to cover

20   the thousands of bases of a gene or other genetic region of interest. The methods of the present invention described below overcome this limitation. At the same time, the present method is distinct from the other fragmentation processes that are limited to screening target nucleic acids for a wide range of potential mutations. Indeed, the appropriate choice of complementary cleavage reactions as described herein allows

25   the determination of the exact location and nature of a genetic variation. Also, it is demonstrated herein that computational protocols are an integral part of the described method. The methods and algorithms are required to deduce, on the basis of the reference sequence(s), the relation between (i) the spectral changes associated with one or more cleavage reactions of a given nature, and (ii) the uniquely defined

30   sequence variations.

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Sequencing reactions according to the methods of the present invention can be multiplexed, *i.e.* used for the simultaneous analysis of multiple non-contiguous target regions [*supra*]. Therefore, the methods can be adapted as a tool for the genome-wide discovery and/or scoring of polymorphisms (*e.g.* SNPs) useful as

5   markers in genetic linkage studies. Indeed, it will be recognized that the unambiguous identification/diagnosing of a number of variant positions is less demanding than full sequencing and that consequently a considerable number of target genomic loci can be combined and analyzed in parallel, especially when their lengths are kept relatively small. The number of markers that can be scored in parallel will depend on the level

10   of genetic diversity in the species of interest and on the precise method used to prepare and analyze the target nucleic acids, but may typically be in the order of a few tens to up to 100 or more with current MS capabilities. The addition of multiplexing to the high-precision and high-speed characteristics of MS constitutes a new marker technology that enables the large-scale and cost-effective scoring of several (tens of)

15   thousands of markers. Some aspects of the application of the present methods to genome-wide genotyping are described in Example 5.

The present invention provides a mass spectroscopy (MS) based nucleic acid sequencing method that overcomes some of the drawbacks inherent in the prior art. In contrast to the previously described methods, the methods of the present

20   invention do not require the generation of a ladder, *i.e.* an ordered set of nested nucleic acid fragments characterized by a common end. Rather, the disclosed methods rely on a combination of complementary fragmentation reactions and the analytical resolution power of MS to improve mass resolution and mass accuracy. The present invention is directed to the use of enzymatic cleavage protocols that result

25   in the generation of cleavage products that range from mono- and dinucleotides to fragments of a few tens of nucleotides that are particularly suited for analysis by MS. According to the present invention, a combination of several different mass spectra, obtained after complementary digestion reactions, coupled with systematic computational analysis allows the survey of a selected nucleic acid or region thereof

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and leads to the unambiguous assignment of both known and previously unknown sequence variations.

The present invention is also directed to methods for the diagnostic sequencing (also referred to as re-sequencing) of all or part of a sample nucleic acid, *i.e.* the determination of the presence, the nature and the location of the sequence variations that occur relative to a related known reference sequence. The sequence variations may either be previously identified or hitherto unknown. Diagnostic sequencing according to the present invention may focus on particular positions in a nucleic acid sequence, *e.g.* when scoring previously known mutations or polymorphisms.

The term "mapping", as used herein, will be understood to include both the characterization, *i.e.* determination of the nature, and the position of the sequence variations.

The terms "target DNA", "target sequence", "target nucleic acid" and the like, as used herein, refer to the sequence region which is to be sequenced or re-sequenced entirely or in part as well as to the nucleic acid material that is actually subjected to one or more complementary cleavage reactions.

The terms "reference nucleic acid sequence", "related sequence", "previously known sequence", and the like, refer to a nucleic acid region, the sequence of which has previously been determined which corresponds to the target. The reference and target sequences may be found to be identical or may differ. The reference sequence need not derive from the same species. In many applications, several different sequence variants will be available as reference. The differences between a target sequence and its reference sequence may be simple (*e.g.*, single nucleotide substitutions, deletions and insertions; microsatellite polymorphisms) or complex (*e.g.*, substitution, insertion, and deletion of multiple nucleotides). In certain situations, one may not know in advance to what reference sequence, if any, the target nucleic acid corresponds. In such situations the interrogated target sequence typically corresponds to a portion of a (much) larger reference sequence and/or to one out of a plurality of different references.

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The terms "unambiguous", "unique", "unequivocal", and the like, are used to indicate that only a single sequence variation or combination of sequence variations can explain the observed mass spectral changes.

The terms "complementary (cleavage) reactions", "complementary cleavages" and the like, as used herein, refer to target nucleic acid digestions characterized by varying specificity [e.g., stringent or relaxed mono- and di-nucleotide specificity; digestion with a combination of reagents; partial cleavage] and/or to digestion alternative forms of the target sequence [e.g., the complementary (+) and (-) strands; incorporation of modified subunits; analysis of variable portions of the target sequence].

The terms "transcript" and "transcription", as used herein, refer to the synthesis of a nucleic acid polymer by means of an RNA polymerase. In addition to canonical subunits (having a 2'-OH group), a transcript may incorporate non-canonical substrates (having any other substituent than a hydroxyl group at the 2'-position). Canonical and non-canonical substrates may contain additional modifications.

The term "genotyping," as used herein, refers to determining the genetic constitution, which is the particular set of alleles inherited by the organism as a whole, or the type of allele found at a particular locus of interest.

The term "expression profiling," as used herein, refers to method(s) for determining the mRNA expression profile of a given cell or a population of cells at a given time under a given set of conditions.

Nucleotides are designated as follows. A ribonucleoside triphosphate is referred to as NTP or rNTP; N can be A, G, C, U or m^{5}U to denote specific ribonucleotides. Likewise, deoxynucleoside triphosphate substrates are indicated as dNTPs, where N can be A, G, C, T, or U. Throughout the text, monomeric nucleotide subunits are denoted as A, G, C, or T with no particular reference to DNA or RNA. When necessary, the nature of the nucleoside monophosphates is clarified by the use of more specific abbreviations such as U, m^{5}U, CMP, and UMP to refer to

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ribonucleotides and dC, dU, dCMP, dUMP and dTMP to indicate deoxynucleotides. Note that T is not an alternative designation for m^5U.

Sequencing via non-ordered sets of specific cleavage fragments

5   The methods of the present invention allow the interrogation every position in a given target sequence without creating a fragment-ladder, *i.e.* a nested set of fragments that share one common endpoint. The method comprises, in part, subjecting one or more target nucleic acids to a set of complementary mononucleotide- and/or dinucleotide-specific cleavages, the products of which are

10   analyzed by mass spectroscopy (MS). A preferred method according to the invention includes the specific cleavage of the one or more target nucleic acids at each nucleotide by way of two or more separate reactions. The digestion products obtained in mononucleotide- and dinucleotide-specific cleavage reactions such as those described herein range from mononucleotides to fragments of a few tens of

15   nucleotides and are particularly well suited for analysis by MS. This aspect of the invention overcomes the technical limitation of the short read lengths encountered when analyzing fragment-ladders under the current MS performance. The mass spectra obtained with the methods do not provide a simple readout of the sequence. Computational approaches provided herein allow the comparative analysis of the

20   obtained spectra with those known or predicted for the related reference sequence.

The ability to detect and map sequence variants based on the non-ordered set of cleavage fragments according to the present invention resides in part in the combination of the various complementary site-specific reactions. For example, one cleavage scheme useful in the practice of the present invention makes use of the

25   mononucleotide-specific ribonuclease-T1 (RNase-T1, G-specific) and RNase-U2 (A-specific; the limited specificity of this enzyme is recognized and will be dealt with below). Both purines (A/G) and pyrimidines (C/T) in a target nucleic acid can be examined by cleaving an RNA copy of the two complementary strands of a target nucleic acid with both enzymes. MS analysis of the fragments generated by only a

30   single mononucleotide-specific reaction would detect the presence of most sequence

variations but only a minority of the mutations - in essence those affecting the nucleotide that is recognized – would also be localized. Since the methods of the present invention examine each of the four bases in a given sequence, each of the twelve possible nucleotide substitutions result in the loss of one cleavage site and the

5   concomitant gain of another cleavage site. This principle is illustrated in Table I for the RNase-T1 and RNase-U2 cleavage reactions on the two complementary transcripts of a hypothetical target nucleic acid. Transitions affect both the RNase-T1 and RNase-U2 cleavage patterns of either the (+) or the (-) strand. As can be seen in Table 1, all transversions change the cleavage pattern of both strands of the transcript:

10   they affect either one of the RNase digests on both strands, or the T1 digest of one strand and the U2 digest of the complementary strand. In addition to altering two cleavage patterns, each single nucleotide substitution also affects the molecular mass of one fragment in each of the remaining two digestion reactions (Table I). In conclusion, complementary cleavage reactions of the present invention results in a

15   high degree of built-in redundancy. Each nucleotide substitution is potentially associated with a maximum of ten differences (data points) with respect to the reference spectrum. The loss and gain of a cleavage site are associated with both the disappearance and appearance of three peaks; two additional peaks undergo a shift as a result of a mass difference. In practice, the 1 Da mass difference between C and

20   U(T) may result in the loss of a significant amount of information (Table I). More particularly, in G- and A-specific cleavage reactions, the C/U transitions may go unnoticed while the observed mass difference may not be unambiguously assigned to a certain transversion. However, in preferred methods of the present invention directed to the analysis of RNA target sequences the method makes use of C and/or U

25   analogs that exhibit more favorable mass differences, thus allowing the unambiguous assignment of the mass difference to a particular transversion. Example 1 and Table I illustrate that 5-methyluridine is an example of such a useful analog [m^5U; R.I. Chemical, Orange, CA; *see also* to Hacia J. *et al.*, *Nucleic Acids Res.* 26: 4975-4982 (1998) for the incorporation of m^5UTP during *in vitro* transcription reactions].

30   Figure 1A shows, by way of example, a 120-nucleotide segment of

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exon 5 of the p53 gene as well as a graphical representation of the digestion products
generated by RNase-T1 and RNase-U2 on an RNA copy of each strand. Figure 1B
displays the size distribution of the base-specific digestion fragments derived from
another exemplary sequence and illustrates that mono-, di- and tri-nucleotides are
5   considerably more numerous than the larger digestion products. This distribution is
expected for mononucleotide specific cleavage reactions that generate fragments with
an average length of four nucleotides. Contrary to the size distribution, the number of
different molecular masses that oligonucleotides can assume rapidly increases with
the size of the fragment. Because of the constrained composition of digestion products
10   (e.g. only one G in the case of RNase-T1), the number of molecular masses of mono-,
di- and tri-nucleotides is limited to 1, 3 and 6, respectively. Consequently, mono-, di-
and tri-nucleotides are often non-informative in the methods of the present invention
because their number exceeds the limited mass space. Figure 1A illustrates that in
certain parts of the target sequence one of the cleavage reactions produces many small
15   fragments due to an over-representation of the recognized nucleotide and,
consequently, yields virtually no information. However using the method of the
present invention, this problem is minimized by the complementary nature of the four
reactions which ensures that the fragments derived from the same region by the other
digestions (interrogating under-represented nucleotides) are correspondingly larger.
20   This indicates a basic attribute of the methods of the present invention. Each of the
four cleavage reactions yields information about a particular mutational alteration (see
Table I) and, in general, the redundancy in this information enables the identification
of the mutation (nature and location) even when part of the information is missing
from the spectra as described above.
25   The methods of the present invention are therefore largely, yet not
completely, sequence-independent and permits the re-sequencing of virtually any
variation. Computer simulations of diagnostic sequencing by the present methods,
more particularly those involving digestion of RNA copies of each strand with the
RNases T1 and U2, have shown that for target sequences of up to three hundred base-
30   pairs ~90% or more of all possible single nucleotide substitutions are associated with

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≥4 data points. Fewer than 1% of the substitutions do not result in spectral changes. More than 95% of all possible single nucleotide substitutions give rise to unique spectral changes and can therefore be unambiguously identified (see Example 1 and Figures 3 and 4).

5   In summary, deduction of the sequence according to the methods of the present invention is based on the integration of the information that resides in a complementary set of 'mass-fingerprints' as well as the previous knowledge about a related reference sequence. The relationship between this multitude of data allows inferring the presence, nature and position of sequence variations in an unambiguous

10   way. It is illustrative of the method that the derivation of the sequence is not critically dependent on the accuracy, *i.e.*, the absolute values of the mass measurements. It is rather the coherent ensemble of mass-shifts and appearances/disappearances of cleavage sites that uniquely define the sequence. The computer simulations, described herein, assumed a resolution of 5 Da or 0.1%, a figure which is well above what can

15   be achieved with state-of-the-art equipment. Also, it should be pointed out that the determination of the correct base composition is limited anyway to short fragments, even in the case of high-precision measurements [*e.g.*, 5-mers in the case of unrestrained sequences and if the measurement has an accuracy of 0.01% or better; Limbach P., *Mass Spectrom. Rev.* 15: 297-336 (1996)]. Other methods in the art,

20   which involve the accurate mass determination to assign the correct base composition to one or more fragments, will generally permit the detection of most sequence variations but not their unequivocal mapping. In these experiments it is generally assumed that a certain experimental observation relates to one particular previously known sequence variation, ignoring the fact that alternative sequence variations can

25   explain the same result.

The present invention encompasses several additional embodiments and aspects described hereinafter and certain other embodiments will be readily apparent to one of ordinary skill in the art.

30   *Target nucleic acid preparation and fragmentation*

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(a) Derivation of target nucleic acid and approaches to cleaving with base-specificity

Nucleic acid molecules can be isolated from a particular biological sample using any of a number of procedures, which are well-known in the art, the particular isolation procedure chosen being appropriate for the particular biological

5   sample. To obtain an appropriate quantity of isolated target nucleic acid on which to perform the methods of the present invention, amplification of the target nucleic acid may be necessary. Examples of appropriate amplification procedures for use in the invention include but are not limited to: cloning [Sambrook *et al., Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press (1989)], polymerase

10   chain reaction (PCR) [Newton C.R. and Graham A., PCR, BIOS Publishers (1994)] and variations such as RT-PCR [Higuchi *et al., Bio/Technology* 11: 1026-1030 (1993)] and allele-specific amplification (ASA), strand displacement amplification (SDA) [Terrance Walker G. *et al., Nucleic Acids Res.* 22: 2670-77 (1994)], and transcription based processes.

15   One embodiment of the present invention is directed to methods for sequencing (re-sequencing, etc.) Nucleic acid comprising the digestion of an RNA copy of each strand of the target nucleic acid with the RNases T1 and U2. One of the advantages of the method is the use of RNA, which exhibits higher sensitivity and better stability in MALDI-MS compared to DNA [Hahner S. *et al., Nucleic Acids Res.*

20   25: 1957-1964 (1997)]. Typically, the first stage of this aspect of the invention involves the amplification of the target nucleic acid by PCR or reverse-transcription followed by PCR. (RT-PCR) This can be achieved with a pair of dedicated primers that incorporate promoter sequences as non-annealing 5'-extensions. In a second stage, these promoters are used for the specific transcription of the adjacent sequences

25   including the target sequences. Preferably, the promoter sequences are small and permit the *in vitro* transcription by a single subunit cognate RNA polymerase such as those deriving from bacteriophage T7, T3 and SP6. Preferred for use in this aspect of the invention are C and/or U analogs that can be incorporated during transcription and that exhibit favorable mass differences [*e.g.* m^{5}U; *supra*]. The use of PCR primers that

30   carry different promoter sequences permits the generation of an RNA copy of both

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strands in two parallel strand-specific transcription reactions. Both strands may also be transcribed from the same promoter sequence: this requires two parallel amplification reactions with only one promoter tagged primer. Alternatively, the *in vitro* transcripts may also be produced from sequences cloned in special purpose

5   vectors such as the pGEM-type vectors available from Promega (Madison, WI) which contain appropriate promoters. The third step further comprises the treatment of the resultant RNA transcripts with one or more complementary mononucleotide-specific RNases (e.g RNase-T1 and RNase-U2), such that each desired position in the target sequence is interrogated. The final step in the process consists of the mass-

10   spectrometric analysis of the RNA fragments resulting from the complementary cleavage reactions and the comparison of the spectra obtained with those of the known reference sequence.

Alternative schemes to prepare target nucleic acid obtained from a biological sample and to subject the target sequence to a set of complementary

15   mononucleotide-specific cleavage reactions are also within the scope of the invention. The target nucleic acid can be DNA, cDNA, any type of RNA, DNA/RNA hybrid, or of mosaic RNA/DNA composition [depending on the ratio of ribo- and deoxyribonucleoside triphosphates (rNTP/dNTP) in the synthesis reaction; Sousa R. and Padilla R., *EMBO J.* 14: 4609-4621 (1995); Conrad F. *et al., Nucleic Acids Res.*

20   23: 1845-1853 (1995)]. The target sequence may also include modifications that are either introduced during or after enzymatic synthesis.

In general, different forms of each target sequence will be prepared so as to be able to perform a complementary set of mono-specific cleavage reactions. The cleavage reactions may be performed enzymatically and/or chemically. The

25   mononucleotide-specificity of the digestion reactions may reside in the cleaving agent (*e.g* RNase T1), in the structure of the target nucleic acid, or in a combination of both. For example, RNase A (specific for both C- and U-residues) can be made monospecific by modifications of the substrate sequence that block the ribonucleolytic action at C or U residues. RNase A cleavage at U residues can in theory be prevented

30   by chemical modification [Simoncsits A. *et al., Nature* 269: 833-836 (1977)]. The

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enzymatic incorporation of nucleotide analogs, most notably those modified at the 2'-hydroxyl group of the ribose is particularly preferred in the practice of the invention. A variety of such analogs have been demonstrated to be substrates for T7 RNA polymerase; e.g. 2'-fluoro, 2'-amino [Aurup H. et al., Biochemistry 31: 9636-9641

5   (1992)], 2'-O-methyl [Conrad F. et al., Nucleic Acids Res. 23: 1845-1853 (1995)], as well as 2'-deoxy NTPs [Sousa R. and Padilla R., EMBO J. 14: 4609-4621 (1995); Conrad F. et al., Nucleic Acids Res. 23: 1845-1853 (1995)]. The above strategy may also be used to improve the specificity of certain RNases such as RNase U2 which is said to cleave GpN phosphodiester bonds in extensive digests [Brownlee G., in

10   "Laboratory Techniques in Biochemistry and Molecular Biology" (Work T.S. and Work E., eds.), North-Holland, Amsterdam, pp 199-200 (1972)]. Mosaic DNA/RNA target sequences that incorporate only one specific rNTP and that can be obtained quite efficiently with particular mutant polymerases [Sousa R. and Padilla R., EMBO J. 14: 4609-4621 (1995); Gao G. et al., Proc. Natl. Acad. Sci. USA 94: 407-411

15   (1997); Bonnin A. et al., J. Mol. Biol. 290: 241-251 (1999)], may allow mono-specific cleavages by alkaline treatment or by digestion with a non-specific RNase such as RNase-I [Meador J. et al., Eur. J. Biochem. 187: 549-553 (1990)].

        Alternative strategies to obtain selective cleavage of target sequences make use of phosphorothioate chemistry. DNA and RNA polymers with

20   phosphorothioate internucleoside linkages in the Rp stereo-configuration are readily synthesized [see Eckstein F., Ann. Rev. Biochem. 54: 367-402 (1985) and references cited therein]. Such phosphorothioate linkages can be specifically hydrolyzed following alkylation [Gish G. and Eckstein F., Nucleic Acids Symp. Ser. pp 253-256 (1987); Gish G. and Eckstein F., Science 240: 1520-1522 (1988)]. Mono-nucleotide

25   specific fragmentation according to this aspect of the invention would require the synthesis of targets making use of one particular α-thio nucleotide triphosphate substrate. Some nucleases (e.g. nuclease P1) cannot hydrolyze Rp phosphorothioate diesters; indirect selective cleavage (at a natural phosphodiester) may thus be obtained with target sequences that incorporate three different αS-dNTPs (or αS-rNTPs).

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(b) Alternative complementary reactions

The performance of the present sequencing methods will be understood by those skilled in the art to be dependent on the following interrelated factors: (1) the length of the region to be sequenced, (2) the resolution of the MS analysis, and (3), to some

5 extent, the sequence itself. The longer the region of interest and, consequently, the larger the number of digestion products, the more important the resolution becomes. Also, the length of the region to be sequenced is directly proportional to the number of single nucleotide substitutions that cannot be unambiguously mapped on the basis of the four base-specific fragmentation patterns only (Example 1; Figure 4). Some

10 sequence motifs are intrinsically difficult to sequence. An example of such a sequence is $CTAGC_1C_2C_3C_4C_5GATC$ (SEQ ID NO: 15), where mutations at C_1 and C_2 cannot be discriminated from the same type of mutations at C_5 and C_4, respectively. Another such sequence is $GAG_1A_2G_3A_4GA$, where G_1->A cannot be discriminated from the G_3->A mutation; similarly, A_2->G and A_4->G cannot be distinguished. Finally, the

15 four mono-nucleotide specific cleavages may also be insufficient to analyze complex sequence variations (see discussion below). Most preferably, therefore, the practicing of the present invention includes a computer-aided simulation of the re-sequencing strategy of the intended region. Such simulation and analysis will reveal possible problematic positions in the sequence and can be used to assess the usefulness of

20 certain additional complementary cleavage reactions as countermeasures to overcome such sequencing difficulties.

One such measure consists of dividing the target region and deriving two or more (partially overlapping) segments (*e.g.*, amplicons) from the sample nucleic acid rather than sequencing the target region as a whole. In addition to setting

25 the length, this allows to exert some control over the composition. This would abrogate problems arising when the region of interest contains a duplicated segment. A second measure consists of carrying out one or more alternative or additional reactions involving target fragments that incorporate one or more modified nucleotides that exhibit different molecular masses such as is described above. Those

30 of skill in the art will know of the existence of a wealth of mass-modified nucleotide

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analogs, many of which are useful and can be reconciled with the enzymatic
procedures of the present method. The nucleotide analogs will differentially affect the
masses of many of the digestion products and will therefore yield a significantly
different spectrum that may reveal the required information. The analogs U and m^5U

5   [*supra*] exemplify this. Simulation studies (which model the present invention) have
indicated that the use of U resolves certain sequence ambiguities observed with m^5U
(data not shown), while overall the latter nucleotide analog results in considerably
fewer sequence ambiguities (*see* Example 1).

Another option consists of performing one or more additional reactions

10   on the complementary strand. Compared to, for example, a G-specific cleavage of one
strand, the C-reaction of the complementary sequence will yield a different set of
fragments characterized by other mass differences. The effect of including reactions
on the complementary strand of the target sequence is therefore similar to the use of
nucleotide analogs.

15   Still another alternative provided by the present invention and which is
useful in obviating the potential problems exemplified above includes using reactions
with alternative specificities of cleavage. For example, partial base-specific cleavage
can be achieved by changing the reaction conditions or by use of a specially prepared
target wherein the cleavable and uncleavable (*e.g.* 2'-modified; *supra*) forms of one

20   particular nucleotide occur randomly. Alternatively, instead of partial base-specific
cleavages, one or more specific digestions characterized by a greater stringency can be
performed (*e.g.* dinucleotide- or relaxed dinucleotide-specificity; *see* below). The
digestion of the target sequence, in double stranded DNA form, with restriction
enzymes is still another alternative provided by the present invention. Double

25   digestion (*i.e.* a combination of two base-specific cleavages) of target nucleic acid
alone or in combination with other digestion methods of the present invention also
represents an informative alternative within the scope of the present invention.

Another informative option within the scope of the present invention
involves the analysis of truncated target sequences. More specifically, cleavage of

30   chain terminated sequences prepared, for example, by incorporation of a particular 3'-

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deoxy nucleotide substrate, will yield spectra that contain additional fragments when compared to the spectrum of the full target nucleic acid and will consequently provide additional information that will, in certain cases, allow a more unambiguous indemnification of sequence variation. This approach will be particularly useful for

5   the characterization of lengthy digestion products or regions containing complex sequence variations.

(c) Alternative complementary reactions: cleavage characterized by a greater than mononucleotide specificity

10   In still another of its embodiments, the method of the present invention also includes nucleolytic processes that are characterized by a dinucleotide- or a relaxed dinucleotide-specificity. Such stringency of cleavage will facilitate the analysis of longer target sequences because the size distribution of the resultant

15   digestion products is even better suited for analysis by MS than fragments with an average length of 4 nucleotides that are generated by mononucleotide-specific cleavage. Useful in this aspect of the invention are, for example, restriction endonuclease reagents capable of cutting DNA at dinucleotide sequences such as those described by Mead D. *et al.*, WO 94/21663 (PCT/US94/03246). RNases that

20   preferentially hydrolyze pyrimidine-adenosine (CA and UA) bonds have also been identified which are useful in the practice of the present invention [*E. coli* RNase-M, Cannistraro V. and Kennell D., *Eur. J. Biochem.* **181**: 363-370 (1989); as in an endoribonuclease isolated from *Saccharomyces cerevisiae*, Stevens A. *et al., J. Bacteriol.* **164**: 57-62 (1985); and as is the *Enterobacter sp.* C-ribonuclease, described

25   by Marotta C. *et al., Biochemistry* **12**: 2901-2904 (1973)]. As disclosed and exemplified in the present invention, the specificity of these enzymes can, if need be, essentially be restricted to CA- or UA-bonds by the use of target nucleic acids that incorporate dUMP (or dTMP) on the one hand and dCMP on the other hand.

Stringent or relaxed dinucleotide-specific cleavage may also be

30   engineered through the enzymatic and chemical modification of the target nucleic acid. By way of non-limiting example, transcripts of the nucleic acid of interest may

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be synthesized with a mixture of regular and α-thio-substrates and the

phosphorothioate internucleoside linkages may subsequently be modified by

alkylation using reagents such as an alkyl halide (e.g. iodoacetamide, -iodoethanol) or

2,3-epoxy-1-propanol. The phosphotriester bonds formed by such modification are

5   not expected to be substrates for RNases. Using this procedure, a mono-specific

RNase, such as RNase-T1, can be made to cleave any three, two or one out the four

possible GpN bonds depending on which substrates are used in the α-thio form for

target preparation. The repertoire of dinucleotide-specific reagents useful in the

practice of the present invention may be further expanded by using additional RNases,

10   such as RNase-U2 and RNase-A. In the case of RNase-A, the specificity may be

restricted to CpN or UpN dinucleotides through the enzymatic incorporation of the 2'-

modified form of the appropriate substrates as described above. For example, to make

RNase-A specific for CpG dinucleotides, a transcript (target) is prepared using the

following substrates: αS-dUTP, αS-CTP, αS-ATP, and GTP. Thus, using the

15   indicated methods described herein, it is possible to engineer all 16 dinucleotide

specificities. However, not all dinucleotide-specific reagents described herein would

be required if the complementary strand of the target nucleic acid is included in the

analysis.

The strategy outlined above makes it possible to prevent cleavage

20   within homopolymer tracts (stretches of A's, G's, C's or T's) by an RNase that is

made specific (or is made specific as described above) for the repeated nucleotide.

Indeed, incorporation of a particular αS-NTP, followed by alkylation, will selectively

prevent cleavage within repeated stretches of that nucleotide, allowing cleavage to

occur at the 3'-side of the last nucleotide in the repeat. Simulation studies, similar to

25   those described in Example 1, have identified this as a particularly useful strategy.

Sequence analysis by digestion of the two complementary strands with RNase-T1 and

RNase-U2 yielded a 5- to 10-fold reduction in the number of ambiguous mutations

when αS-GMP and αS-AMP were incorporated in the respective transcripts. These

studies also suggest that the selective blockage of cleavage within repeats is

30   accompanied by a relatively small increase in the average length of the digestion

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products, thereby resulting in considerably less loss of information.

Those of skill in the art will also readily recognize variations or alternatives in certain aspects of the fragmentation methods described herein. Such alternatives or variations encompassed by the present invention include but are not

5   limited to:

1.   the use of other or additional RNases (alone or in combination) having similar or alternative specificities;

2.   the use of mutant or chemically modified RNases with useful characteristics vis-

10   à-vis the methods of the present invention [see for example, Loverix S. *et al.*, *Nature Struct. Biol.* 5: 365-368 (1998) for an RNase T1 mutant that prefers the phosphorothioate analog over the natural phosphodiester substrate; *see also* Contreras R. and Fiers W., *FEBS Lett.* 16: 281-283 (1971) for the production of limited digests with a chemically modified RNase];

15

3.   the use of other nucleotide analogs that exhibit different masses and/or reactivities, including nucleotides that incorporate alternative isotopes; and

4.   alternative specific fragmentation methods, either chemical [Maxam A. and

20   Gilbert W., *Proc. Natl. Acad. Sci. USA* 74: 560-564 (1977); Richterich P. *et al.*, *Nucleic Acids Res.* 23: 4922-4923 (1995)], or enzymatic.

Multiplex reactions

In another embodiment, the methods of the present invention are

25   directed to the simultaneous sequence determination of at least two non-contiguous regions in a sample nucleic acid. In contrast to traditional sequencing methods that generate a fragment-ladder (*i.e.* a nested set of fragments that share a common endpoint), the strategies outlined herein are equally useful for multiplex sequencing. Multiplex sequencing, according to the present invention, generally involves the co-

30   amplification of selected regions of target nucleic acids. This can be achieved by

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using sets of dedicated primer pairs which flank or are co-terminal with a target
nucleic acid to be amplified. Alternatively, the preparation of the multiple target
nucleic acids comprises the concomitant amplification of restriction fragments derived
from the sample nucleic acid. Some approaches are illustrated and exemplified in
5   Example 5. A special case of multiplex sequencing consists of the simultaneous
analysis of the two complementary strands of a double stranded target nucleic acid.

In yet another embodiment, the methods of the present invention can be
used for the simultaneous sequence determination of the corresponding target
region(s) of at least two biological samples. A sequence variation in one out of a pool
10   of analogous target nucleic acids may go unnoticed when analyzing conventional
sequence ladders by means of gel electrophoresis. With the present methods, a
sequence variation will, as a rule, yield one or more distinct peaks in the various
complementary mass spectra. This feature should allow the detection of mutations at
a significantly lower ratio of mutant to wild-type allele and therefore permit the
15   analysis of larger pools. The ability to pool renders the present methods useful for the
discovery of sequence variations across particular target regions in a given population.
For this application, typically 5-10 samples may be combined. In case the mutations
have previously been identified, considerably more samples, e.g. several tens, can be
combined. The characteristics that render the present method useful for the analysis
20   of sample pools make the method also effective for the analysis of heterozygous
samples (i.e., an equimolar mix of two alleles).

Mass spectrometric methods

Mass-spectrometric methods useful in the practice of the present
25   invention include ionization techniques such as matrix assisted laser desorption
ionization (MALDI) and electrospray (ES). These ion sources can be matched with
various separation/detection formats such as time-of-flight (TOF; using linear or
reflectron configurations), single or multiple quadrupole, Fourier transform ion
cyclotron resonance (FTICR), ion trap, or combinations of these as is known in the art

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of mass spectrometry. [Limbach P., *Mass Spectrom. Rev.* **15**: 297-336 (1996); Murray K., *J. Mass Spectrom.*, **31**: 1203-1215 (1996)].

Because the present methods generally require the analysis of complex oligonucleotide fragment mixtures, the MALDI approach, mostly resulting in singly 5   charged molecules, is preferred over ES where significant multiple charging will further increase the number of spectral peaks. For the desorption/ionization process, numerous matrix/laser combinations can be employed.

Sequence determination of simple versus complex variations

10   In another embodiment, the methods of the present invention are directed to the diagnostic sequencing of one or more target nucleic acids that, in comparison with a related reference nucleic acid, incorporates a sequence variation other than a single nucleotide substitution. Such a sequence variation can involve the deletion or insertion of one or more nucleotides as well as the substitution of multiple 15   nucleotides.

Similar to single nucleotide substitutions, the insertion or deletion of a single nucleotide represents a simple sequence variation whose analysis using methods of the present invention is straightforward. Both of these types of sequence variations are associated with a characteristic set of (maximum nine) changes in the 20   four complementary mononucleotide-specific fragmentation patterns. It will be understood that the methods of the present invention, similar to other sequencing methods, may not unambiguously locate the point of insertion or deletion when it concerns one nucleotide in a stretch of identical nucleotides. This, however, may be taken into consideration when performing a computer assisted analysis of whether the 25   observed spectra relate in a unique way to a specific sequence variant in accordance with the practice of the present invention.

Analysis of a microsatellite DNA [also referred to as VNTR (variable number tandem repeat) or SSR (simple sequence repeat)] represents a special case whose analysis is readily achieved using the methods of the present invention. 30   Although multiple nucleotides are involved with VNTRs or SSRs, the interpretation

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of the spectral changes on the basis of the known reference sequence is rather simple and the polymorphism (an altered number of repeat units) may readily be characterized.

The methods of the present invention may also be used to analyze more complex sequence variations such as those where multiple nucleotides are affected either through insertion, deletion, substitution or a combination thereof. The analysis of a number of double and triple mutants is described below in Example 3d. Multiple substitutions within a target sequence are also expected to be accompanied by a characteristic number of spectral changes. This number depends on whether the substitutions are adjoining or separated, as well as on the intervening sequence in case the mutations are separated. Single nucleotide substitutions, isolated by a sequence that contains at least one A, G, C, and T, are each associated with 10 spectral differences as outlined above. In general, the analysis of complex sequence variants will require (elaborate) computational approaches. One possible algorithm involves the comparison of the experimentally observed spectra with those generated on the basis of all possible sequences in the short region to which the sequence variation is confined. Such an algorithm will identify the sequence variant or, in case of ambiguities, the different matching sequences. This procedure illustrates that the present methods may be applied to the *de novo* sequencing of short regions of a target sequence. It will be recognized that, in practice, the experimental observations will not only set the boundaries but will also define the length of the variant region such that the algorithm need not consider insertions or deletions. Additional experimentally derived information, such as the absence of a particular nucleotide, can further limit the sequence space the algorithm has to explore. In particular applications, the complex sequence variants may be previously known and may thus be part of the set of reference sequences. In such cases, the experimentally observed spectra may be directly correlated to those predicted for the reference sequences. There would however still be a need to compute whether such correlation is unique. The advantage of previous knowledge is that the experimental approach can be adapted such that the

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output information indeed relates uniquely to the potentially occurring complex sequence variations.

Computer algorithm

The present invention, in part, rests on the insight that computational analysis of the spectra obtained in a set of complementary cleavage reactions, and comparison of these data with the computationally predicted spectral changes from the known reference sequence, as illustrated herein, is an important step in the unambiguous determination of the presence, the nature and the location of sequence variations. More specifically, the computational approaches to simulate the experiment illustrated herein are necessary to determine whether a unique relation exists between the spectra obtained and a particular sequence variation. Accordingly, one aspect of the present invention contemplates a method which utilizes a computer algorithm or method capable of computing the spectral differences resulting from one or more nucleotide differences between the target nucleic acid and the reference nucleic acid, the method and algorithm comprising subjecting the reference nucleic acid and sequence variants thereof (*i.e.*, target nucleic acid having nucleotide differences) to the different base specific cleavages to generate oligonucleotide fragments, computing the mass of each oligonucleotide fragment, generating the mass spectra of the oligonucleotide fragments from the reference nucleic acid and the sequence variants thereof for each of the base specific cleavage reactions, and matching these computationally derived mass spectra with the spectra obtained experimentally in the different base specific cleavage reactions.

In one preferred embodiment the computer algorithm is designed to systematically compute the spectra of all possible simple nucleotide variations of the reference nucleic acid, including but not limited to all possible single nucleotide substitutions, deletions and insertions. Since most of the genetic diversity found in living organisms involves single nucleotide variations, most of the experimentally observed sequence variations can be identified with the methods and algorithms of the present invention, meaning that one or more matches may be found between the

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observed spectra and the computationally derived mass spectra. In case a unique match is found, the sequence variation in the target nucleic acid is unique. When more than one match is found between spectra, the sequence variation cannot be established unambiguously.

5   It will be obvious to the person skilled in the art that different approaches may be used for performing the computational analysis, such as, but not limited to, performing the computational analysis on the complete reference sequence, or performing a serial computational analysis on segments of the reference sequence using, for example, a sliding window. The latter approach will enable the

10   identification of different sequence variants occurring in different parts of the reference sequence.

In another embodiment, the methods and computer algorithms of the present invention are designed to explore all possible nucleotide sequences in a limited segment of the reference sequence. Such methods and algorithms may be used

15   when the preceding approach fails to give a match, demonstrating that the sequence variation does not correspond to a simple nucleotide variation in the reference nucleic acid. This may be the case when more than one nucleotide change occurs within a short region, such that one or more cleavage products contain multiple nucleotide alterations. The region corresponding to these cleavage products can then be explored

20   further by computing the spectra for all possible sequence permutations and determining the matching sequence. It is anticipated that given sufficient computing power, such methods and algorithms may be used for *de novo* sequencing using mass spectral data generated according to the present invention.

25   ***Applications of the present methods***

The methods of the present invention are particularly well suited for rapidly and accurately re-sequencing nucleic acids from a variety of biological sources including, but not limited to, plants, animals, fungi, bacteria and viruses. Re-sequencing implies the detection and mapping of both previously known as well as

30   unknown sequence variations (*e.g.* mutations and polymorphisms) relative to a related

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reference sequence. One of the most notable distinctions with respect to conventional gel-electrophoretic analysis of fragment ladders, is that generally each particular sequence (variation) results in a distinct and characteristic set of (mass) peaks. This feature makes the present methods effective for the reliable scoring of heterozygous

5   samples, the simultaneous sequencing of multiple target regions from a single biological sample (*i.e.*, multiplexing), as well as the simultaneous analysis of the analogous regions from different samples (*i.e.*, pooling). The use of pools of individual samples should permit the cost-effective identification of previously unknown sequence variations in a population. This aspect of the invention properties

10   makes the present methods valuable for clinical and public health studies. Very often such studies rely on samples (*e.g.*, saliva, blood, swabs, paraffin-embedded tissue, biopsy material) that are cellularly and genetically heterogeneous and, consequently, require assays that can detect mutations at a low ratio of mutant over wild-type allele.
       An additional advantage of the present methodology is that it can be

15   tuned (by reducing the number of complementary cleavage reactions) such that the diagnostic sequencing is limited to particular positions in a target nucleic acid, a feature useful for the unambiguous scoring of previously identified mutations or polymorphisms. The processes described herein can be used, for example, to diagnose any of the more than 3000 genetic diseases currently known (*e.g.*,

20   hemophilias, thalassemias, Duchenne Muscular Dystrophy, Huntington's Disease, Alzheimer's Disease and Cystic Fibrosis) or genetic defects yet to be identified. In addition, certain DNA sequences may predispose an individual to any of a number of diseases or conditions such as diabetes, artherosclerosis, obesity, various autoimmune diseases and cancer (*e.g.*, colorectal, breast, ovarian, lung). Depending on the

25   biological sample, the diagnosis for a genetic disease or genetic predisposition can be performed either pre- or post-natally using the methods of the present invention. Re-sequencing of nucleic acids derived from infectious organisms using the methods of the present invention may reveal the basis of pathogenicity and may also be useful to identify the variation(s) that cause drug-resistance. For example, mutations in the

30   protease/reverse transcriptase region of the human immunodeficiency virus (HIV)

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have been implicated in the decreased sensitivity towards the antiviral activity of protease and reverse transcriptase (RT) inhibitors. The re-sequencing of the nucleic acid encoding these viral domains is therefore of special interest to monitor disease progression (*see* Example 1). Similarly, sequencing, according to the present

5   invention, may be useful to determine the antibiotic-resistance phenotype of certain bacteria [*e.g. Mycobacterium tuberculosis*; Head S. *et al., Mol. Cell. Probes* 13: 81-87 (1999); Troesch A. *et al., J. Clin. Microbiol.* 37: 49-55 (1999)].

In other embodiments, the present methods are directed to the identification and classification of target nucleic acids. Analyses according to the present

10 invention characterize nucleic acids at a level essentially equal to sequence determination. Therefore, interrogated unknown sequences may be unambiguously identified by comparison of the obtained mass spectra with those known or predicted for a plurality of reference sequences. In this exercise, novel sequences that have no matching reference database sequence may also be found. The use of the methods for expression profiling

15 (*i.e.*, the analysis of cDNA libraries) as well as whole-genome sequencing is exemplified in Example 6 and 7, respectively. Other applications include the determination of identity or heredity (*e.g.*, paternity or maternity).

Kits for practicing the invention

20 Kits for diagnostic sequencing of one or more target nucleic acids in a sample are also provided. In preferred embodiments, such kits comprise one or more reference nucleic acids, various reagents for sequence specific cleavage protocols, and computer algorithm(s). Such kits may optionally also contain nucleic acid amplification reagents. Additionally, the kits may contain reagents for the preparation of modified

25 nucleic acids, including but not limited to modified nucleotide substrates. The kits may also contain buffers providing conditions suitable for certain enzymatic or chemical reactions. In addition, the kits may contain reagents, such as solid supports, for purposes of isolating certain nucleic acids and preparing nucleic fragments for mass spectrometric analysis.

30 The foregoing aspects of the invention are illustrative and should not be

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construed to limit the invention as set out in the appended claims. Variations in some aspects as well as alternative procedures will be readily recognized by one of ordinary skill in the art.

Example 1 describes modeling the diagnostic sequence analysis of a 1200
5   base-pair region of HIV-1 using methods of the present invention.

Example 2 describes methods for base-specific cleavage by modifying the nucleic acid template to be cleaved.

Example 3 illustrates the diagnostic sequencing of the RNase-T1 coding region according to the methods of the present invention.

10   Example 4 illustrates the analysis of a ~1000 base-pair nucleic acid.

Example 5 illustrates the use of the present invention for genotyping, including multiplex genotyping.

Example 6 illustrates the use of the present invention for transcription profiling.

15   Example 7 illustrates the use of the present invention for whole genome resequencing.

EXAMPLE 1

Modeling the Diagnostic Sequence Analysis of a 1200 Base-pair
20   ### Region of HIV-1

The methods of the present invention have been utilized on a 1200 base-pair sequence derived from human immunodeficiency virus type 1 (HIV-1; HXB2 isolate; Genbank accession number K03455; position 2161 to 3360). This sequence was used as a model in computer simulations to examine the overall
25   performance of the method, as well as the occurrence of ambiguities. The selected region encompasses the entire protease gene and the first ~270 codons of reverse transcriptase [compare with Hertogs K. *et al., Antimicrob. Agents Chemother.* **42**: 269-276 (1998)]. The genotyping/re-sequencing of this domain of clinical isolates of HIV is of special interest in order to monitor the emergence of drug resistance-
30   associated mutations. Single as well as multiple changes have been implicated in the

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decreased sensitivity towards the antiviral activity of protease and RT inhibitors [Hertogs K. *et al., Antimicrob. Agents Chemother.* **42**: 269-276 (1998); Schinazi R. *et al., Int. Antivir. News* **4**: 95-107 (1996) and references cited therein].

5   The principal objective of the computer simulation was to examine the performance of the re-sequencing method for detecting and mapping SNPs. To this end we have performed computational simulation analyses in which we have systematically mutated each nucleotide one by one in the 1200 base-pair sequence. For each mutation we have calculated the molecular masses of the cleavage products that would be generated from a given segment of the sequence in the different four

10   RNase digestion reactions, namely upon RNase-T1 and RNase-U2 cleavage of the (+) and (-) strands. The comparison of these masses with those of the reference cleavage products from the original sequence identifies the masses of the diagnostic fragments associated with each mutational change, *i.e.*, fragments that either appear or disappear as a result of the mutation. The underlying assumption in this analysis was that in

15   order to be measurable, the fragment must have a molecular mass different from those of the other cleavage products generated in the same reaction. Furthermore, we have assumed that the resolution of the mass spec analysis is limited to mass differences larger than either 5 Da or 0.1%. In other words fragments whose mass difference with other fragments in the same digest is smaller than 5 Da or 0.1% were not scored in the

20   analysis. The quantitative aspects of a mass spectrum (*i.e.* peak heights) were not considered in the present simulation study. For each mutational change we have computed the number of fragments that are diagnostic for the presence of the mutation. Mutational changes were scored as detectable when there was at least one diagnostic fragment (showing a spectral change). In addition, we have examined

25   whether the mutational changes can also be mapped unambiguously. To this end we have compared the sets of diagnostic fragments associated with each mutation. Mutations that yield unique sets of fragments can be mapped unambiguously, while mutations that give the same sets cannot be distinguished from one another.

     In a first simulation analysis we have computed the fraction of SNPs

30   that may be detected and mapped using respectively 1, 2 and 4 RNase digestion

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reactions. To this end we have performed a systematic single nucleotide substitution simulation on a 200-base-pair segment of the HIV sequence. For each of the four different RNase digestion reactions [RNase-T1 and RNase-U2 cleavage of the (+) and (–) strands] we have calculated the number of detectable diagnostic fragments and have analyzed whether these fragments are unique for each mutation. The results summarized in Figure 2 show that in each of the single RNase digest reactions a large fraction (55% to 85%) of the mutations are detected. In contrast, only a small fraction (20% to 30%) of these mutational variations can be mapped unambiguously. The principal reason is that many different mutational changes result in the same mass differences. The fraction of mutations that can be mapped increases to around 60% to 70% when the data of two RNase digest reactions are combined. The further combination of the data from the four different cleavage reactions allows 96% of the mutational changes to be positioned unambiguously and illustrates the advantages of the methods of the present invention. Close inspection of the sequence ambiguities reveals that about half of these involve C to U (or conversely A to G) transitions. Because the difference in molecular mass between C and U residues is only 1 Da, the mass difference in the cleavage products of the strand carrying the pyrimidine base is too small to be detectable. Consequently one might expect that these mutational changes may become detectable when using m^5U instead of U. Computational simulations using m^5U on the same 200 base-pair sequence shows that the fraction of mutations that can be mapped unambiguously increases to 98%. Consequently all further simulations are based on the use of the analog m^5U. These results demonstrate that the four mononucleotide-specific RNase digests are both necessary and sufficient for re-sequencing of most sequences with a high degree of accuracy.

It will be obvious that the quality of the sequences obtained with the methods of the invention will be strongly influenced by the size of the sequence segments that are examined. Indeed, the larger the size of the segment, the larger the statistical chance that certain relevant diagnostic fragments may coincide with other cleavage products generated in the same reaction. We have therefore performed a systematic single nucleotide substitution simulation analysis on the 1,200 base-pair

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HIV sequence using different size segments, namely 100, 200, 300 and 600 base-pairs. In each simulation a total of 3,600 single mutational substitutions was analyzed. For each of the four different RNase digest reactions both the number and the patterns of the measurable diagnostic fragments were computed using the

5   detection limits described above. Figure 3 shows the distribution of the number of diagnostic fragments obtained with the 3,600 mutational changes in the four different analyses. The results clearly indicate that a larger percentage of the single nucleotide substitutions is associated with fewer diagnostic spectral changes when using larger segments of DNA.

10   In each simulation we determined both the number of detectable SNPs as well as the fraction of SNPs that can be mapped unambiguously. The results of the computational simulations summarized in Figure 4 show that almost all the mutational changes are detected in the four different analyses. Of the 3,600 SNPs, the number that escaped detection were respectively 0, 1, 3 and 9 using 100 base-pair, 200

15   base-pair, 300 base-pair and 600 base-pair segments, respectively. In contrast, the fraction of mutational variations that can be mapped unambiguously decreases much more when using longer segments. While only 1% of the SNPs are ambiguous when analyzing 100 base-pair segments, that fraction increases to almost 10% with 600 base-pair segments. Close inspection of the ambiguities shows that the majority of

20   these involve nearby (often adjacent) pairs of identical bases where the analysis can determine the nature of the mutation but fails to identify which of the bases is changed.

               In conclusion, the results of the simulations show that the methods of the invention are effective for re-sequencing and that even large segments may be

25   used when only a limited number of positions need to be analyzed. Also, it appears that in most cases a computer-aided simulation study will be essential in the experimental design as well as the data interpretation when using the methods of the present invention. Most importantly, the simulations will indicate whether spectral changes are unambiguously linked to particular sequence variations.

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EXAMPLE 2

Base-Specific Cleavage by Modification of the Template

The present example illustrates that the specificity of cleavage by a nucleolytic reagent may be further confined through the modification of the target

5   template such that particular phosphodiester bonds resist cleavage. More particularly, it is demonstrated that RNase-A, which normally cleaves at the 3'-side of both C- and U-residues, becomes mononucleotide-specific when the target incorporates the 2'-deoxy analog of one of these nucleotides. A region of the plasmid vector pGEM3-Zf(+) (Promega, Madison, WI), encompassing the multi-cloning site as well as the

10   phage T7 promoter sequences, was used as a model (*see* Figure 5).

The first step towards the sequence analysis according to the present invention involved the amplification of the 158 base-pair test sequence. The reaction was carried out in a total volume of 50 l using 12.5 pmol each of the forward and reverse primer, 200 M of each dNTP, 0.25 l Taq DNA polymerase (5 U/ l; Promega,

15   Madison, WI), 1.5 mM MgCl$_2$ and a buffer supplied with the enzyme. After an initial incubation at 94°C for 2 min, 40 cycles of the following temperature program were performed: 94°C for 30 sec, 50°C for 30 sec, and 72°C for 15 sec. The sample was kept an additional 15 min at 72°C and then chilled. The PCR reaction product was purified (High Pure PCR Product Purification Kit; Roche Diagnostics Belgium,

20   Brussels, Belgium) and subsequently used for transcription of one specific strand. A mutant T7 RNA polymerase (T7 R&DNA™ polymerase; Epicentre, Madison, WI) with the ability to incorporate both dNTPs and rNTPs was used in the transcription reactions. In addition to a transcription with the regular ribonucleotide substrates, one reaction was performed where CTP was replaced by dCTP, while in two more

25   separate transcriptions either dUTP or dTTP replaced UTP. The transcription reactions were run in a 50 l volume containing: 40 mM Tris-Ac (pH 8.0), 40 mM KAc, 8 mM spermidine, 5 mM dithiothreitol, 15 mM MgCl$_2$, 1 mM of each rNTP, 5 mM of dNTP (in these cases the appropriate NTP was excluded), ~40 nM DNA template (~2 pmol), and 250 units T7 R&DNA™ polymerase. Incubation was

30   performed at 37°C for 2 hours. After transcription, the full-length T7 *in vitro*

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transcripts (118 nucleotides) were purified by allowing them to anneal to the 5'-
biotinylated form of the complementary reverse PCR primer (Figure 5) followed by
capture of the biotinylated annealing products onto streptavidin-coated magnetic
beads. To this end, 50 pmol biotinylated reverse primer was added to the transcription

5   reactions. The mixtures were first incubated 5 min at 70°C and, subsequently, ~30
min at room temperature. Then, a slight excess of Sera-Mag™ streptavidin magnetic
microparticles [Seradyn Inc, Indianapolis, IN; resuspended in 50µl of 2 M NaCl, 20
mM Tris-HCl (pH 8.0), 2 mM EDTA] was added and the resultant mixture incubated
at room temperature for 30 min with agitation. A magnetic particle collector (MPC;

10   Dynal, Oslo, Norway) was used to collect the beads, remove the supernatant and,
subsequently, to wash the beads three times with 100 1 100 mM $(NH_4)_3$-citrate. The
beads were finally resuspended in 3 1 25 mM $(NH_4)_3$-citrate containing 0.5 g bovine
pancreas RNase-A (50 U/mg; Roche Diagnostics Belgium, Brussels, Belgium) and
incubated at room temperature for about 30 min to digest the transcripts to

15   completion. 1 1 of this RNase reaction was removed and added to 5 1 matrix solution.
This 1:1 acetonitrile:H_2O matrix solution is saturated with 3-hydroxypicolinic acid
(~100 mg/ml), and further contains 25 mM $(NH_4)_3$-citrate, (occasionally) 2 pmol/ 1 of
an oligonucleotide serving as an internal standard, and cation-exchange beads in
$(NH_4)^+$-form (Dowex 50W-X2; Sigma, Saint-Louis, MO) to minimize the presence of

20   sodium and potassium adducts. After incubating the mixture at room temperature for
15 min, 1 1 was put on the sample plate and allowed to dry. Mass spectra were
collected using a Reflex III mass spectrometer (Bruker Daltonik GmbH, Bremen,
Germany).

        The RNase-A cleavage products predicted for each of the four

25   transcripts are shown in Table II. Note that the mass calculation of the predicted
fragments assumes a 3'-phosphate group and not the 2',3'-cyclic phosphate
intermediate of the cleavage reaction. Overall, the experimentally obtained spectra
(Figure 6) are in excellent agreement with the predictions. The absence of some of
the smallest 3-mers (Figure 6A and 6C) may be related to the mass-gate that was

30   applied to eliminate the non-informative mono- and di-nucleotide digestion products.

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The predicted 3'-proximal fragment TGTTTC (1830, 1 Da) is only poorly ascertained in Figure 6C, *i.e.*, the spectrum deriving from the dU-transcript. This result, along with other observations, suggests that fragments with a relatively high dU-content are detected with a significantly lower sensitivity using the present MS methodology.

5   The 2817 Da peak in Figure 6D corresponds to the double protonated form of the added oligonucleotide. Some of the expected fragments cannot be resolved because they have an identical composition. Also, the digestion products of the regular transcript that differ by one Da only (*e.g.* the difference between CMP and UMP; Table II) cannot be seen as distinct peaks in Figure 6A. In total, the data convincingly

10   demonstrate that RNase-A behaves as a C-specific RNase when dTTP or dUTP is substituted for UTP, and as a U-specific reagent when dC rather than C is incorporated into the substrate transcripts. This high level of nucleobase specificity is achieved even under the over-digestion conditions used in the present Example.

The protocol described in the present Example is illustrative and certain

15   modifications and variations will occur to the skilled artisan. The immobilization of the transcripts represents an easy means to prepare the material for MS analysis, *e.g.*, removal of all other reaction components and exchange of Na^+ and K^+ counter-ions for $(NH4)^+$ (note that the subsequent RNase digestion does not require any reagents that are 'incompatible' with MS). While other methods, such as chromatography, may be

20   used to prepare the transcripts or the derived digestion products for MS analysis, the present method is favorable in that it is readily amenable to automation and high-throughput analysis. In repeat experiments, yielding essentially the same results as described herein, the transcripts were digested in water and ~15 nanoliter of these digests was directly applied onto a Spectrochip™ (Sequenom Inc., San Diego, CA) for

25   analysis by MALDI-TOF-MS.

EXAMPLE 3

Diagnostic Sequencing of the RNase-T1 Coding Region

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The present example illustrates the application of the methods of the invention to the re-sequencing of a portion of the RNase-T1 coding region. We selected the RNase-T1 coding region because of the availability of a collection of site-directed mutants [Steyaert J., *Eur. J. Biochem.* 247: 1-11 (1997)] which had

5   previously been sequenced using the classical dideoxy chain termination method. The wild-type and mutant sequences, used in the present example, are shown in Figure 7.

a. Analysis of the wild-type RNase-T1 sequence

The experiments were performed essentially as described in Example 2.

10   First, the selected wild-type RNase-T1 target sequences were amplified by PCR with the following primers:

5'-CCGGATATAAACTTCACGAAGACGG   (forward)   (SEQ ID NO: 16)

5'-GATAGGCCATTCGTAGTAGGGAGAGC   (reverse)   (SEQ ID NO: 17)

The resultant amplicon was subsequently re-amplified using either a forward or a

15   reverse primer that incorporates the T7 promoter site as a 5' non-annealing extension
(*see* Figure 7A):

5'-TAATACGACTCACTATAGGGCGACTTCACGAAGACGG (forward)

(SEQ ID NO: 18)

5'-TAATACGACTCACTATAGGGCGAATTCGTAGTAGGGAGAGC (reverse)

20   (SEQ ID NO: 19)

Subsequently, each of the resultant promoter-appended amplicons was used as template in two separate transcription reactions. The T7 R&DNA polymerase (Epicentre, Madison, WI) was used to prepare transcripts that incorporate dCMP or dUMP instead of respectively CMP and UMP (referred to as the dC- and dU-

25   transcripts). The transcription reactions were carried out as described in Example 2, except that each rNTP was present at 2 mM and incubation was performed overnight at 37°C. The four full-length T7-transcripts were purified by annealing with a biotinylated oligonucleotide that matches with the transcript 3'-end (*i.e.* the biotinylated form of either the forward or the reverse PCR primer used in the first

30   amplification step) and subsequent capture onto streptavidin microparticles. After

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extensive washing with $(NH_4)_3$-citrate, the transcripts were eluted. The beads were resuspended in 3 l of water and kept at 90°C for 2 min, immediately followed by collection of the beads with the magnet and transfer of the supernatant to a fresh tube. Then, the obtained amplified target nucleic acids were digested to completion by the

5   addition of 1 l of 100 mM $(NH_4)_3$-citrate containing RNase-A. Finally, the reaction products were analyzed by MALDI-TOF-MS.

A graphical representation of the spectra is shown in Figure 8A-D. The predicted degradation products are listed in Table III. As with the pGEM3-Zf(+) transcripts the obtained spectra are in good agreement with the predictions. A few

10   peaks that are most likely the result of double protonation were also observed (see Figure 8B). The T-reaction on the (-) strand suggests the occurrence of transcripts with an extra non-template encoded nucleotide at the 3'-end [Milligan J. *et al.*, *Nucleic Acids Res.* 15: 8783-8798 (1987)]. Indeed, in addition to the expected 3'-terminal fragment, a prominent peak is observed that coincides with the same

15   fragment containing an extra G-residue (Figure 8D and Table III). The absence of the expected 3'-terminal fragment from the C-reaction on the (+) strand (1153 Da; Figure 8A) may be explained by this same phenomenon. In this case, cleavage of the 3'-extended transcript would occur and result in the 3'-phosphorylated (rather than the 3'-OH) form of the predicted fragment, a product which would coincide with another

20   fragment of the same digestion (1233.7 Da; Table III).

b. Analysis of selected RNase-T1 single point mutations

Four single nucleotide substitutions were chosen (mutant #1, #2, #3, and #4 in Figure 7B). Each of the mutant sequences was analyzed as described for the

25   wild-type RNase-T1 coding region (Example 3a). The results are summarized in Table IV. Table IV shows, for each mutation, which 5 fragments of the wild-type RNase-T1 reference sequence are affected by the mutation as well as the 5 fragments that are mutation-specific. It also shows which changes are missing, and consequently on how many, out of the ten theoretical data points, the mutation identification is

30   actually based. Spectral changes are missing because they involve fragments that are

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too small (<3-mer) or not unique. Also, a few fragments were not experimentally observed, *e.g.*, one 3-mer as well as the largest fragments with a mass of ≥9,8 Kda. Of particular interest are the results concerning mutation #2. These results indeed best illustrate the present invention. In this particular case, all four mono-nucleotide

5   specific cleavage reactions result in the detection of a mutation, *i.e.* one will notice that the sequence differs from the wild-type RNase-T1 coding region. However none of these reactions, when taken alone, leads to the unambiguous mapping of the mutation. The C-reaction on the (+) strand results in a new fragment of 1947 Da. Not only the single nucleotide mutation #2 can explain the creation of such a 6-mer

10   [composition = $A_3G(dU)C$]. For example, this is also the case for a double mutation that converts the sequence CTACTAC into CAAGTAC (*see* Figure 7); the TAC peak will not be lost because of the presence of a third such 3-mer. The T-reaction on the (+) strand results in a spectrum where the mass of one fragment has increased by 56 Da when compared to the reference spectrum. This suggests the replacement of a dC

15   by a G. Because the cleavage product contains three dC residues, it is not possible to position the substitution. The C-reaction on the (-) strand is at first sight the most informative; a large reference fragment is affected by the cleavage. The sequence of the fragment $(GTAG_1TT\text{---}TG_2GATC)$(SEQ ID NO: 20) is however such that both the $G_1\text{->}C$ and the $G_2\text{->}C$ mutation can explain the observed products of 9814 Da and

20   1289 Da [composition = GA(dU)C]. Finally, the T-reaction on the (-) strand is the least informative and the appearance of a peak of 944 Da [A(dC)U] can be explained in many different ways. An A(dC)U-fragment is, for example, generated by substitution of the T_1-residue for a C in the sequence stretch TAT_1TT (*see* Figure 7). In conclusion, mutation #2 exemplifies that in some cases the nature and position of a

25   sequence variation may only be determined by a combination of at least two different complementary cleavage reactions.

c. Analysis of a mixture of wild-type and mutant RNase-T1 sequences

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The analyses shown in Table IV can be used to simulate experiments where equimolar mixtures of the wild-type RNase-T1 sequence and one of the single nucleotide substitutions are examined. In such cases, which mimic heterozygotic genotypes, the spectra contain a number of novel fragments in addition to all those

5   derived from the (wild-type) reference sequence. The characterization and location of the mutation/polymorphism is therefore necessarily based on the novel fragments only. Unambiguity requires that the novel fragments are sufficient to uniquely define the mutation. Those of skill in the art will realize that zygosity determination is straightforward using the present methods because each allele is associated with a

10   distinct set of peaks.

We performed a number of experiments where on particular single nucleotide mutant (*e.g.*, mutant #3; Figure 7B) was mixed with wild-type RNase-T1 such that the mutant allele was present at the following fractions: 1:2. 1:5, 1:10, 1:20, 1:50, 1:100, 1:200, 1:500 and 1:1000. the experiment mimics the analyses of pools of

15   samples characterized by different allele frequencies. First, equivalent quantities of the wild-type and mutant target sequences were synthesized by PCR amplification using conditions where the primers are limiting and completely consumed. After mixing the two amplicons in the desired ratios, the material was re-amplified. Then, transcripts of the (-) strand were prepared and digested as described above, except that

20   transcriptions were performed using all four nucleotide triphosphate substrates in the ribo-form (rNTPs) and that cleavage was carried out with RNase-T1 instead of RNase-A. Each of the digestion reactions was measured 5 times. Cleavage with the RNase-T1 enzyme generates a polymorphic 15-mer fragment which reads: AAAUCAAAACCUUCG(SEQ ID NO: 21), where the underlined residue is changed

25   to A by mutation #3 (*refer to* Figure 7A and 7B). The mass of the wild-type and the mutant fragment is 4807,91 Da and 4830,95 Da, respectively; the mutation causes a shift of 23 Da. We found that there was an excellent linear correlation between the allele frequencies and the relative peak heights (R^2=0,97) and that the peak associated with the mutant allele could still be identified with confidence when it represented 5-

30   10% of the material. It should be noted that in other experiments the minimum ratio

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of mutant over wild-type allele that can be detected might be significantly lower. Indeed, in the present example, the reliable detection of the 'mutant peak' was somewhat encumbered by the occurrence of an extra peak as evidenced by the control spectrum recorded for the wild-type target nucleic acid. This extra peak may possibly

5   be attributed to a low level of Na^+-adduct of the wild-type fragment (22 Da mass shift). In all, the latter data indicate that homologous target nucleic acids can be pooled and analyzed simultaneously; in addition to revealing certain sequence variations, the methods of the present invention may permit the allele frequencies to be estimated among the pool of biological samples. While diagnostic sequence

10   determination as disclosed herein relies primarily on the appearances and disappearances of peaks as well as peak shifts, the present example indicates that certain quantitative aspects of a spectrum (e.g., peak height and peak area) can be included in the sequence analysis and yield complementary valuable information.

15   *d. Analysis of RNase-T1 multiple mutants*

The methods of the present invention are not limited to the analysis of single nucleotide substitutions. Complex variations can also be sequenced. Table IV lists the spectral changes that are predicted to be associated with a number of RNase-T1 multiple mutants, more particularly double and triple mutants (mutant #5, #6, #7,

20   and #8 in Figure 7B). As described above, multiple mutants are associated with a characteristic number of spectral changes. In the case of multiple substitutions, with no deletions or insertions involved, the number of affected reference fragments is always identical to the number of novel fragments. For double mutants the number of spectral changes ranges from 12, in case the mutations are adjoining (mutant #5), to a

25   maximum of 20, in case the mutations are separated by a sequence that contains at least one A, G, C, and T. In the latter case, the double mutant is to be treated as two concurrent but independent single nucleotide substitutions. Triple mutants are associated with a minimum of 14 spectral changes (mutant #7). As with single nucleotide substitutions, not all the theoretical spectral changes can or may be

30   observed and part of the information will be lost. In the vast majority of the cases

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however a systematic computational analysis, based on the obtained spectra and the reference nucleic acid sequence(s), can unambiguously identify and locate the sequence variations.

5   **EXAMPLE 4**

Mass Spectrometric Analysis of a ~1000 Base-Pair Region

The methods of the invention are designed to overcome the limitation of the short read lengths encountered with current MS-based sequencing methodologies that involve the analysis of fragment-ladders. One can envision that,

10 depending on the application, target regions of several hundred or even a few 1000 base-pairs can be analyzed. The present example demonstrates that a large number of oligonucleotide fragments can be analyzed simultaneously by the methods of the present invention and that, consequently, the detection platform does not impose a limit on the methodology.

15   Following the scheme presented in Example 2, a 1012 base-pair region of the plasmid vector pGEM3-Zf(+) (Promega, Madison, WI) was amplified and the resultant amplicon, subsequently, used for preparation of a 972 nucleotides long *in vitro* T7 transcript (see Figure 5). The transcript incorporated dCMP instead of CMP such that a U-specific cleavage could be performed by RNase-A. The cleavage

20 products predicted for this transcript, are listed in Table V. Figure 9 shows the most relevant parts of the experimentally obtained spectrum. The primary conclusion from the experimental data is that complex mono-nucleotide specific digestion reactions, consisting of >200 cleavage products, can be analyzed by mass spectrometry. The vast majority of the about 67 predicted distinct peaks are readily identified. Only a

25 few of the 4-mer fragments are not or barely detectable. It also appears that in the present experiment the assignment of some peaks requires the assumption that (at least a portion of) certain digestion products contains a $2',3'$-cyclic phosphate instead of a $3'$-phosphate group. Such peaks differ from the parent peaks by -18 Da. It is well known that cyclic phosphates result from the transesterification cleavage reaction and

30 that these intermediates get hydrolyzed in a slower second reaction step.

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EXAMPLE 5

Genotyping

The methods of the present invention are also useful for the diagnostic

5   sequencing of multiple non-contiguous regions of a sample nucleic acid. This renders

the present methods useful for the genome-wide discovery as well as the routine

scoring of polymorphisms (*e.g.* SNPs) and mutations at multiple loci in genomic

DNA. Such multiplex genotyping is conceptually no different than re-sequencing;

both require that alterations are characterized and positioned unequivocally. Similar to

10   experiments involving a single target sequence described above, a computer

simulation can be performed to find out which ones of the observed spectral changes

is uniquely linked to particular genomic alterations. Since multiplex genotyping only

requires the identification/diagnosing of a number of variant positions, it will be

recognized by those of skill in the art that (i) the complexity (*i.e.* the combined length)

15   of the multiple target sequences may be significantly greater than in the case of full

re-sequencing, and (ii) a single specific cleavage reaction may often suffice for both

allele and zygosity identification. Applications which involve the use of two

sequence-specific cleavages that each positively identify one of the two alternative

forms of a series of bi-allelic SNPs are also possible using the methods of the present

20   invention. For example, many C to T transitions, the most common type of point

mutations and polymorphisms in human, may be easily scored by a combination of C-

and T(U)-specific reactions. It is worth mentioning that heterozygous samples

analyzed using gel-electrophoretic sequencing are often difficult to identify with

confidence. With the methods described herein, the detection of heterozygosity is

25   unambiguous because of the presence of both the wild-type and the mutation specific

set of mass spectral peaks.

Multiplex genotyping will generally involve the co-amplification of

genomic regions. In the case of previously known SNP genetic markers, co-

amplification of selected loci can be achieved by using dedicated primer pairs [Wang

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et al., Science **250**: 1077-1081 (1998)]. Alternatively, a more generic approach can be adopted for both the discovery and the subsequent routine scoring of a set of SNPs where the preparation of target sequences comprises the concomitant amplification of multiple short restriction fragments derived from the sample nucleic acid. This

5   'random sampling' method may be particularly useful with organisms that have a high polymorphism content (*e.g.*, more than 1 SNP in 100 base-pairs). This co-amplification can be achieved by ligating to the ends of the restriction fragments adaptor sequences that incorporate the target sites for a single PCR primer pair. In this approach, the average size of the amplicons must be small such that the majority

10   incorporates ≥1 SNP while, additionally, the total number of the amplicons must be sufficiently small so that their combined length is amenable to analysis by the present methods. These requisites can be met by the appropriate choice of restriction enzymes and the use of methods that permit the selective amplification of discrete subsets of restriction fragments [Vos P. *et al., Nucleic Acids Res.* **23**: 4407-4414

15   (1995); Zabeau M. and Vos P., EP 0534858 (1993); Kikuya Kato, *Nucleic Acids Res.* **23**: 3685-3690 (1995)] and as described herein. For example, a first restriction enzyme that cleaves rarely in the genome under study can be combined with a second reagent that generates fragments with an average size of about 100 base-pairs (*e.g.*, a combination of two enzymes with tetra-nucleotide recognition sites). The number of

20   fragments edged by the two different restriction sites should preferably be less than 100,000; a suitable subset of these can readily be amplified by the use of selective primers [Vos P. *et al., Nucleic Acids Res.* **23**:4407-4414 (1995)]. In addition, a PCR protocol, characterized by a highly shortened elongation time, can be used such that the amplification of short fragments is strongly favored thereby further reducing the

25   number and the average size of the amplicons. During the selective co-amplification of genomic fragments or in a subsequent PCR step, a first primer can be used that attaches a full promoter sequence (*e.g.*, one deriving from bacteriophage T7, T3 or SP6; *supra*) to the amplicons. The second strand may be synthesized by extension of a primer that contains a ribonucleotide residue at, for example, the penultimate

30   position. Following PCR amplification, the primer sequences can be removed from

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this second strand by RNase digestion, and the resultant truncated strand transcribed with the aid of the first primer. This procedure minimizes the common sequences that are connected to the target restriction fragments.

5   **EXAMPLE 6**

cDNA Library Analyses - Transcription Profiling

Diagnostic sequencing will, generally, be performed on a defined nucleic acid, *i.e.* one will know to what reference sequence the target nucleic acid corresponds. However, the re-sequencing methods according to the present invention

10   can also be used to identify or classify certain sequences. In such experiments, the interrogated nucleic acid (*e.g.* a random clone of DNA) will typically correspond to an unknown portion of a (much) larger sample sequence or represent one out of a plurality of nucleic acids present in a biological sample, or a combination of both. The mass spectra derived from the unknown nucleic acid are compared to those known or

15   predicted for the related reference sequence(s), or portions thereof. Note that, in this type of experiments, some of the interrogated target sequences need not necessarily have their counterparts in the reference sequences, and vice versa. It will be realized that sequence identification according to the present methods may, at the same time, reveal possible sequence variations. Interrogated sequences may thus be classified as

20   identical to one of the database sequences, as a variant of such as a reference sequence or as novel in case no matching sequence is found.

It should be recognized that analyses that involve at least the four complementary mono-nucleotide specific cleavage reactions identify unknown sequences with a resolution essentially equal to sequence determination. At the same

25   time, the MS-based methods described herein allow fast data acquisition and are amenable to high-throughput. Therefore, the present methods are useful to identify and catalogue nucleic acids at an unprecedented scale and speed. One application consists of the analysis of cDNA libraries for the purpose of: (i) the assembly of unigene libraries (*i.e.* the identification/removal of replicate clones), (ii) the

30   identification of novel genes or novel variants of previously identified genes, and (iii)

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transcription profiling. The speed and throughput of the present method should permit the processing of more clones and, hence, a more in depth analysis of a cDNA library.

A variety of methods are known in the art for transcription profiling, i.e. the analysis of the transcription in both qualitative and quantitative terms. In one
5   method, the expressed-sequence-tag (EST) approach, the mRNA population is assessed by partial sequencing of randomly selected cDNA clones. Global changes in gene-expression patterns are deduced from the EST ratios among two compared cDNA libraries [Lee N. et al., Proc. Natl. Acad. Sci. USA 92: 8303-8307 (1995)]. The methods described herein may be used to catalogue expressed genes with a similar
10   level of resolution but considerably higher speed and throughput. First, a library of unidirectionally cloned cDNAs is constructed in a vector that permits transcription of the inserted sequences. Preferably, the 3'-end of the cDNAs is located adjacent to the promoter. Template for transcription can be prepared by amplification of the promoter-cDNA cassette using a pair of vector-specific primers. Alternatively, vector
15   DNA is prepared and cleaved at a restriction site within the vector close to the 5'-end of the inserted cDNA (e.g. ~25 base-pairs). Preferably, the restriction site at which the templates are cleaved should have a low occurrence frequency within the cDNAs under study. Run-off transcripts, synthesized from PCR product or digested vector, are characterized by a common 3'-end, consisting of vector sequences, which allows
20   the isolation of full-length transcripts as described in Example 2. An alternative strategy involves treatment of the vector DNAs with a restriction reagent such that not only all templates are digested at the cDNA 5'-end but that a vast majority is also cleaved within the cDNA at some distance from the 3'-end (e.g. a few hundred base-pairs). The restriction reagent may be a single enzyme or a combination of two or
25   more restriction enzymes. Ligation of an adaptor to the digestion product(s) [see Vos P. et al., Nucleic Acids Res. 23: 4407-4414 (1995)] can be considered so as to obtain full-length transcripts with a common 3'-end enabling their isolation as described in Example 2. However, transcripts that incorporate a biotin group at the 5'-end may also be prepared [Hahner S. et al., Nucleic Acids Res., 25: 1957-1964 (1997)],
30   providing an alternative means for their immobilization. Digestion within the cDNAs

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is an attractive option in that different partial cDNAs deriving from the same transcript are made congruent by this procedure and thereby facile to identify. The full-length run-off transcripts are finally subjected to complementary sequence-specific cleavage reactions, and the resultant digestion products analyzed by MS as

5   disclosed herein.

Those of skill in the art will recognize the advantages of the transcript profiling method outlined above. Comparable to the EST approach, cDNAs are identified at the sequence-level, *i.e.* the ultimate level of resolution. Thus, while the method involves fragmentation of the interrogated nucleic acid, its level of resolution

10   far exceeds that attained by fingerprinting techniques [Prashar Y. and Weissman S., *Proc. Natl. Acad. Sci. USA* **93**: 659-663 (1996); Bachem C. *et al., The Plant Journal* **9**: 745-753 (1996); Ivanova N. and Belyavsky A., *Nucleic Acids Res.* **23**: 2954-2958 (1995); Liang P. and Pardee A., *Science* **257**: 967-971 (1992)]. In contrast to hybridization-based approaches [Schena M. *et al., Science* **270**: 467-470 (1995);

15   Wodicka L. *et al., Nature Biotechnology* **15**: 1359-1367 (1997)] the method can identify both known and previously unknown sequences. Also, it should prove faster then methods requiring gel-electrophoretic fractionation.

EXAMPLE 7

20   ### Whole-Genome Re-Sequencing

In the past couple of years the technology for sequencing entire genomes, especially those of microorganisms, has come to maturity. More than 50 microbial genomes are scheduled to be completed by the year 2000, and the benefits emerging from this vast body of knowledge are rapidly becoming clear [Clayton R. *et*

25   *al., Curr. Opinion Microbiol.* **1**: 562-566 (1998)]. It seems clear that sequencing entire microbial genomes is becoming routine and that microbial genetics is entering the era of 'comparative genomics'. Knowledge of the complete genome sequence is the ultimate tool in phylogenetic analyses, allows gene/functional diversity studies, and fundamentally changes the manner in which research is conducted in an

30   organism. At the present time, a substantial portion of each new genome sequence has

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no database match. One may expect to see a greater proportion of orthologous genes in the future, when the microbial species diversity is better represented. At that point, when most of the sequences generated will be similar to already known sequences, global genome analyses could be performed rapidly, accurately, and cost-effectively

5   using a re-sequencing strategy as described herein rather than by *de novo* sequencing methods. Similar evolutions may be anticipated outside the bacterial genetics field where genome projects for many (model) organisms are ongoing or have already been finished (*e.g.*, *Drosophila melanogaster*, *Caenorhabditis elegans*, human, mouse, *Arabidopsis thaliana*, and rice).

10   The methods of the present invention may be readily adapted to the re-sequencing of entire (bacterial) genomes or megabase nucleic acid regions. This may be accomplished with the use of a shotgun approach that involves the sequence analysis of unselected subclones that harbor random fragments according to the methods of the present invention. The assembly of all the independent, random

15   sequences is fundamentally different from that in a *de novo* sequencing project [Fleischmann R. *et al.*, *Science* 269: 496-512 (1995)] because of the availability of a reference sequence that serves as a scaffold. The assembly into a single complete sequence comes down to matching each set of experimentally obtained spectra with a portion of the reference sequence. The computational approaches required to

20   accomplish this are similar to those that are needed for the analysis of cDNA libraries, outlined in Example 6. In both cases one does not know in advance the reference sequence, if at all existing, for a given interrogated target region. It should be noted, however, that the present shotgun approach might be even more demanding in terms of computational power because of the undefined ends of the segments. At the same

25   time, the algorithms must be capable of mapping the variations that occur between the target and the reference sequence. It is expected that a shotgun approach with its built-in redundancy (*i.e.*, most sequences will be covered several-fold) should prove useful for the comprehensive comparison of a pair of related genomes. An alternative for the shotgun approach strategy consists of the analysis of clones from one or more libraries

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of restriction enzyme fragments or the analysis of defined amplicons generated with locus specific primer pairs.

While the present invention has been described in terms of the preferred embodiments, it is understood that variations and modifications will occur to those skilled in the art. Therefore, it is intended that the appended claims cover all such equivalent variations which come within the scope of the invention as claimed. All of the references cited herein are expressly incorporated by reference.

Table I: Detection of the twelve possible point mutations that can occur in DNA by the methods of the present invention. Each substitution is associated with the loss (- sign) and gain (+ sign) of a cleavage site. In addition, each mutation affects the mass of two digestion products as indicated. Mass differences shown in bold face result from the incorporation of m^5U in both transcripts (see text for details).

Mutation		RNase T1		RNase U2	
(+) strand	(-) strand	(+) transcript	(-) transcript	(+) transcript	(-) transcript
transitions					
A->G	T->C	+	-1 Da **-15 Da**	-	-1 Da **-15 Da**
G->A	C->T	-	+1 Da **+15 Da**	+	+1 Da **+15 Da**
T->C	A->G	-1 Da **-15 Da**	+	-1 Da **-15 Da**	-
C->T	G->A	+1 Da **+15 Da**	-	+1 Da **+15 Da**	+
transversions					
A->C	T->G	-24 Da	+	-	+39 Da **+25 Da**
C->A	G->T	+24 Da	-	+	-39 Da **-25 Da**
T->G	A->C	+	-24 Da	+39 Da **+25 Da**	-
G->T	C->A	-	+24 Da	-39 Da **-25 Da**	+
T->A	A->T	+23 Da **+9 Da**	-23 Da **-9 Da**	+	-
A->T	T->A	-23 Da **-9 Da**	+23 Da **+9 Da**	-	+
C->G	G->C	+	-	+40 Da	-40 Da
G->C	C->G	-	+	-40 Da	+40 Da

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Table II: RNAse-A digestion products predicted for four different pGEM3-Zf(+)

derived transcripts. The ≥3-mer fragments are ranked according to their

molecular masses. The regular transcript was prepared with rNTP

substrates. Transcripts that incorporate dTMP, dUMP, or dCMP are

denoted as dT-, dU-, or dC-transcript. Fragments containing a 5'-

triphosphate (5'ppp-) are indicated.

regular transcript		dT-transcript		dU-transcript		dC-transcript	
Fragments	expected mass (M+)	fragments	expected mass (M+)	fragments	expected mass (M+)	fragments	expected mass (M-)
CAT	959.6	TGC	973.6	TGC	959.6	CCT	903.5
AAT	983.6	GAC	998.6	GAC	998.6	CAT	943.6
AGC	998.6	ATGC	1302.8	ATGC	1288.8	CAT	943.6
AGC	998.6	AAGC	1327.8	AAGC	1327.8	AAT	983.6
GAC	998.6	GAGC	1343.8	GAGC	1343.8	AGT	999.6
AGT	999.6	AGGC	1343.8	AGGC	1343.8	GGT	1015.6
GGC	1014.6	5'ppp-GGGC	1599.7	TTGGC	1594.9	AGCT	1288.8
GGT	1015.6	TTGGC	1623.0	5'ppp-GGGC	1599.7	AGCT	1288.8
GGT	1015.6	ATAGC	1632.0	ATAGC	1618.0	CGGT	1304.8
AAAT	1312.8	GGTAC	1648.0	GGTAC	1634.0	AAAT	1312.8
AAGC	1327.8	TGTTTC	1886.2	TGTTTC	1830.1	GAGT	1344.8
GAAT	1328.8	GAATTC	1936.2	GAATTC	1908.1	CACCT	1521.9
GAGC	1343.8	GTAATC	1936.2	GTAATC	1908.1	GGCGT	1650.0
AGGC	1343.8	ATGGTC	1952.2	ATGGTC	1924.1	AGAGT	1674.0
GAGT	1344.8	TAGAGTC	2281.4	TAGAGTC	2253.4	CGACCT	1867.1
5'ppp-GGGC	1599.7	GGGGATC	2338.4	GGGGATC	2324.4	CGAGCT	1923.2
AGAGT	1674.0	TAAATAGC	2594.6	TAAATAGC	2566.6	GCAAGCT	2252.4
GGGGAT	2035.2	TATAGTGTC	2889.8	TATAGTGTC	2833.7	GCAGGCAT	2597.6
		TTGAGTATTC (SEQ ID NO. 22)	3194.0	TTGAGTATTC (SEQ ID NO. 22)	3123.9	5'ppp-GGGCGAAT	2893.5
						ACCCGGGGAT (SEQ ID NO 23)	3232.0

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Table III:　RNase-A digestion products predicted for the dU- and dC-transcripts of the (+) and (-) strands of the RNase-T1 coding region. Only the $\geq$3-mers are shown. Cleavage of the dU-transcript is C-specific. Likewise, the T-reaction is performed on the dC-transcript. Two fragments, shown in italics, assume the occurrence of 3'-extended transcripts (refer to Example 3).

(+) strand / C-reaction	[M+H]⁺	(+) strand / T-reaction	[M+H]⁺
TTC	904.5	ACT	943.6
TAC	943.6	GAT	999.6
TAC	943.6	CCCT	1192.7
TAC	943.6	GGAT	1344.8
AAC	982.6	CCAAT	1562.0
AAC	982.6	GGCCT	1594.0
GAC	998.6	GAGCT	1634.0
TATC-OH3'	1153.7	GAAACT	1947.2
TATC-p3'	*1233.7*	ACGAAT	1947.2
TTAC	1233.7	ACGAAGGT	2637.6
AATTC	1562.9	ACAACAACT	2838.8
5'ppp-GGGC	1599.7	5'ppp-GGGCGACT	2853.5
AAATAC	1931.2	ACCCACACAAAT	3746.4
GAAGAC	2002.2	(SEQ ID NO: 25)	
TGTGAGC	2269.4	CACGAAGACGGT	3890.4
GAATGGC	2308.4	(SEQ ID NO: 26)	
GGTGAAAC	2637.6		
TGTTGGATC	2849.7		
GAAGGTTTTGATTTC (SEQ ID NO: 24)	4723.8		
(-) strand / C-reaction	**[M+H]⁺**	**(-) strand / T-reaction**	**[M+H]⁺**
TTC	904.5	CGT	959.6
TTC	904.5	CGT	959.6
GTC	959.6	CGT	959.6
AAC	982.6	AGT	999.6
5'ppp-GGGC	1599.7	AGT	999.6
AAAAC	1641.0	CCGG-OH3'	1207.8
AGTTTC	1869.1	*CCGGG-OH3'*	*1553.0*
GAATTC	1908.1	GGAT	1344.8
AGAGAAATC	2950.8	GGGT	1360.8
GTGAAGTTTATATC (SEQ ID NO: 27)	4417.7	GAAGT	1674.0
GTAGTAGGGAGAGC (SEQ ID NO: 28)	4637.8	CACCGT	1867.1
GTAGTTGTTGTATTTGTGTGGGTAAGAATTGGATC	11123.7	AAGAAT	1987.2
(SEQ ID NO: 20)		CAAAACCT	2509.6
		CCAACAGT	2525.6
		5'ppp-GGGCGAAT	2893.5
		AGGGAGAGCT	3328.0
		(SEQ ID NO: 29)	
		CACAGAGAAAT	3569.2
		(SEQ ID NO: 30)	

Table IV: Spectral changes associated with single and multiple mutations in the RNase-T1 coding region.

REACTION	AFFECTED REFERENCE FRAGMENTS			NOVEL FRAGM.	
	SEQUENCE	[M+H]+	Comments	[M+H]+	Comments
mutation #1 [A->T on (+) strand / T->A on (-) strand]					
(+) / C	TTAC	1233,7	not unique	1194,7	
(+) / T	ACCCACACAAAT (SEQ ID NO: 25)	3746,4		325,2 3417,1	< 3-mer
(-) / C	GTAGTTGTTGTATTTGTGTGGGTAAGAATTGGATC (SEQ ID NO: 20)	11123,7	not observed	11162,7	not observed
(-) / T	GGGT AAGAAT	1360,8 1987,2		3352,1	
mutation #2 [C->G on (+) strand / G->C on (-) strand]					
(+) / C	AAC TAC	982,6 943,6	not unique not unique	1.947,2	
(+) / T	ACAACAACT	2.838,8		2.894,8	
(-) / C	GTAGTTGTTGTATTTGTGTGGGTAAGAATTGGATC (SEQ ID NO: 20)	11.123,7	not observed	1.288,8 9.813,9	not observed
(-) / T	AGT	999,6	not unique	943,6	
mutation #3 [A->T on (+) strand / T->A on (-) strand]					
(+) / C	GAAGGTTTTGATTTC (SEQ ID NO: 24)	4723,8		4684,8	
(+) / T	ACGAAGGT	2637,6		1618,0 1015,6	
(-) / C	TTC	904,5	not unique	943,6	not observed
(-) / T	CAAAACCT T	2509,6 325,2	< 3-mer	2838,8	
mutation #4 [A->T on (+) strand / T->A on (-) strand]					
(+) / C	GAAGGTTTTGATTTC (SEQ ID NO: 24)	4723,8		4684,8	
(+) / T	ACGAAGGT	2637,6		1288,8 1344,8	not unique
(-) / C	TTC	904,5	not unique	943,6	
(-) / T	T CGT	325,2 959,6	< 3-mer not unique	1288,8	
mutation #5 [AC->CG on (+) strand / GT->CG on (-) strand]					
(+) / C	AAC TAC	982,6 943,6	not unique not unique	653,4 1.288,8	< 3-mer
(+) / T	ACAACAACT	2.838,8		2.854,8	not resolved
(-) / C	GTAGTTGTTGTATTTGTGTGGGTAAGAATTGGATC (SEQ ID NO: 20)	11.123,7		1.288,8 9.868,9	
(-) / T	AGT T	999,6 325,2	not unique < 3-mer	1.288,8	

Table IV (continued)

REACTION	AFFECTED REFERENCE FRAGMENTS			NOVEL FRAGM.	
	SEQUENCE	[M+H]⁺	Comments		Comments
mutation #6 [AAA->CAG on (+) strand / TTT->CTG on (-) strand]					
(+) / C	AAATAC	1931,2		324,2	< 3-mer
				1618,0	
(+) / T	ACCCACACAAAT (SEQ ID NO: 25)	3746,4		3722,3	
(-) / C	GTAGTTGTTGTATTTGTGTGGGTAAGAATTGGATC (SEQ ID NO: 20)	11123,7		4104,4	
				7108,3	
(-) / T	AT	654,4	< 3-mer	943,6	
	T	325,2	< 3-mer		
	T	325,2	< 3-mer	1015,6	
	GT	670,4	< 3-mer		
mutation #7 [AAT->GCG on (+) strand / ATT->CGC on (-) strand]					
(+) / C	AATTC	1562,9		669,4	< 3-mer
				959,6	
(+) / T	CCAAT	1562,0		1883,1	
	T	325,2	< 3-mer		
(-) / C	GTAGTTGTTGTATTTGTGTGGGTAAGAATTGGATC (SEQ ID NO: 20)	11123,7		8904,3	
				669,4	< 3-mer
				1634,0	
(-) / T	AAGAAT	1987,2		3601,2	
	T	325,2	< 3-mer		
	GGAT	1344,8			
mutation #8 [AAC,AAC, TAC -> AAG,AAT,TTC on (+) strand / GTA,GTT,GTT -> GAA,ATT,CTT on (-) strand]					
(+) / C	AAC	982,6		2856,7	
	AAC	982,6			
	TAC	943,6	not unique		
(+) / T	ACAACAACT	2838,8		2605,6	
				325,2	< 3-mer
	ACGAAGGT	2637,6		325,2	< 3-mer
				2308,4	
(-) / C	GTAGTTGTTGTATTTGTGTGGGTAAGAATTGGATC (SEQ ID NO: 20)	11123,7		2237,4	
				8888,3	
(-) / T	CGT	959,6	not unique	1947,2	
	AGT	999,6	not unique		
	GT	670,4	< 3-mer	614,4	< 3-mer

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Table V: U-specific cleavage of a 972 nucleotides long T7 transcript. The predicted
digestion products, 222 in total, are grouped according to their
composition. An asterisk indicates those peaks for which a companion
cyclic phosphate reaction intermediate is observed (Figure 9). The largest
fragment is absent from the obtained spectrum; a few other cleavage
products appear as minor peaks and are labeled 'weak'.

Composition	M+H	Length	Number	Remarks
T	325,2	1	47	
CT	614,4	2	11	
AT	654,4	2	14	
GT	670,4	2	15	
C_2T	903,5	3	4	
ACT	943,6	3	3	
CGT	959,6	3	7	
A_2T	983,6	3	5	
AGT	999,6	3	1	
G_2T	1015,6	3	4	
C_3T	1192,7	4	2	*
AC_2T	1232,7	4	5	*
C_2GT	1248,7	4	4	
A_2CT	1272,8	4	3	
ACGT	1288,8	4	6	
CG_2T	1304,8	4	5	
A_3T	1312,8	4	1	weak
A_2GT	1328,8	4	1	weak
AG_2T	1344,8	4	5	
AC_3T	1521,9	5	1	
C_3GT	1537,9	5	2	
A_2C_2T	1562,0	5	2	
AC_2GT	1578,0	5	2	*
C_2G_2T	1594,0	5	7	*
A_2CGT	1618,0	5	1	weak
ACG_2T	1634,0	5	3	*
CG_3T	1650,0	5	3	
A_3GT	1658,0	5	2	
A_2G_2T	1674,0	5	2	
G_4T	1706,0	5	1	*
C_5T	1771,1	6	1	*
C_4GT	1827,1	6	1	*
AC_3GT	1867,1	6	2	*
C_3G_2T	1883,1	6	2	
A_3C_2T	1891,2	6	1	

Table V (continued)

AC_2G_2T	1923,2	6	2	*
C_2G_3T	1939,2	6	1	
A_4GT	1987,2	6	1	
AC_4GT	2156,3	7	1	
C_4G_2T	2172,3	7	1	
A_2C_3GT	2196,3	7	1	
AC_3G_2T	2212,3	7	2	
A_3C_2GT	2236,4	7	1	
$A_2C_2G_2T$	2252,4	7	2	
C_2G_4T	2284,4	7	1	
A_3CG_2T	2292,4	7	1	
A_2CG_3T	2308,4	7	2	
ACG_4T	2324,4	7	1	
AC_5GT	2445,5	8	1	*
$A_2C_2G_3T$	2597,6	8	1	
A_4CG_2T	2621,6	8	1	
A_2CG_4T	2653,6	8	1	
A_4C_3GT	2854,8	9	1	
A_6C_2T	2878,8	9	1	
$A_2C_3G_3T$	2886,8	9	1	
A_2CG_4T (5'ppp-)	2893,6	8	1	
$A_3C_2G_3T$	2926,8	9	1	
C_8GT	2983,9	10	1	weak *
$A_2C_5G_2T$	3119,9	10	1	
$A_3C_3G_3T$	3216,0	10	1	*
$A_2C_3G_4T$	3232,0	10	1	
$A_3C_2G_4T$	3272,0	10	1	
$A_2C_2G_5T$	3288,0	10	1	
A_5C_5T	3417,1	11	1	
$A_5C_3G_2T$	3529,2	11	1	
A_6CG_3T	3625,2	11	1	
AC_3G_7T	3938,4	12	1	
$A_2C_7G_3T$	4043,5	13	1	
$A_3C_5G_3T$	4139,6	12	1	
$A_5C_3G_4T$	4219,6	13	1	
$A_4C_2G_6T$	4291,6	13	1	
$A_3C_8G_3T$	4661,9	15	1	
$A_5C_4G_5T$	4854,0	15	1	
$A_9C_3G_4T$	5536,4	17	1	
$A_6C_6G_6T$	6106,8	19	1	
$A_4C_7G_{13}T$	8154,0	25	1	
$A_{13}C_8G_{10}T$	10370,5	32	1	not observed
			$\Sigma = 222$	

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We Claim:

1.   A method for sequence analysis of one or more target nucleic acids for which a reference nucleic acid sequence is known, the method comprising the
5   steps of:

(a)   deriving from one or more biological samples the one or more target nucleic acids;

(b)   subjecting the one or more target nucleic acid to complementary cleavage reactions with one or more cleavage reagents thereby
10   generating cleavage products;

(c)   performing mass spectroscopical analysis on the cleavage products generated in step (b) to obtain one or more mass spectra; and

(d)   comparing the one or more mass spectra of the cleavage products of the one or more target nucleic acids obtained in step c) with a known or
15   predicted mass spectra for the reference nucleic acid sequence, and deducing therefrom, by systematic computational analysis, all or part of the nucleotide sequence of the one or more target nucleic acids, and comparing the deduced nucleic acid sequence with the reference nucleic acid to determine whether the one or more target nucleic acids have the same sequence or a different sequence from the reference
20   nucleic acid.

2.   The method of claim 1 wherein the nucleic acid sequence difference that is determined in step d) is a deletion, substitution, insertion or combinations thereof.
25

3.   The method of claim 1 wherein the biological sample is derived from organism selected from the group consisting of eukaryotes, prokaryotes, and viruses.

30   4.   The method of claim 1 wherein the target nucleic acid is selected

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from the group consisting of single stranded DNA, double stranded DNA, cDNA, single stranded RNA, double stranded RNA, DNA/RNA hybrid, and DNA/RNA mosaic nucleic acid.

5   5.   The method of claim 4 wherein the one or more target nucleic acids is derived by one or more consecutive amplification procedures selected from the group consisting of *in vivo* cloning, polymerase chain reaction (PCR), reverse transcription followed by the polymerase chain reaction (RT-PCR), strand displacement amplification (SDA), and transcription based processes.

10

6.   The method of claim 5 wherein the one or more amplified target nucleic acids are RNA transcripts generated from a single stranded or a double stranded target nucleic acid by a process comprising the steps of:

            (a)   linking operatively an expression control sequences to the
15   one or more target nucleic acids; and

            (b)   transcribing one or both strands of the one or more target nucleic acid of step a) using one or more RNA polymerases that recognize the transcription control sequence on the one or more target nucleic acids.

20   7.   The method of claim 6 wherein the transcription control sequence is selected from the group consisting of an eukaryotic transcription control sequence, a prokaryotic transcription control sequence, and a viral transcription control sequence.

25   8.   The method of claim 7 wherein the prokaryotic transcription control sequence is selected from the group consisting of T3, T7, and SP6 promoters.

9.   The method of claim 8 wherein the RNA polymerases which utilize the T3, T7, or SP6 promoters are either wild type or mutant RNA polymerases,

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the mutant polymerases being capable of incorporating into the RNA transcript non-canonical substrates with a 2′-substituent other than a hydroxyl group.

10.   The method of claims 4, 5, 6, 7, 8 or 9 wherein the target nucleic acid is derived using one or more modified nucleoside triphosphates selected from the group consisting of mass modified deoxynucleoside triphosphates, mass modified dideoxynucleoside triphosphates, and mass modified ribonucleoside triphosphates.

11.   The method of claim 10 wherein the modified nucleoside triphosphates are modified on the base, sugar, and/or the phosphate moiety, and are introduced through an enzymatic step, chemically, or a combination of both.

12.   The method of claim 10 wherein the modification consists of 2′-substituents other than a hydroxyl group on the nucleotide triphosphates.

13.   The method of claim 10 wherein the modification consists of phosphorothioate internucleoside linkages or phosphorothioate internucleoside linkages further reacted with an alkylating reagent.

14.   The method of claim 10 wherein the modification consists of a methyl group on C5 of the uridine-5′-monophosphate subunits.

15.   The method of claim 10 wherein the modifications alter the specificity of cleavage by the one or more cleavage reagents and/or the mass and/or the length of the cleavage products.

16.   The method of claims 11, 12, 13 or 14 wherein the modifications alter the specificity of cleavage by the one or more cleavage reagents and/or the mass and/or the length of the cleavage products.

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17.   The method of claim 1 wherein the complementary cleavage reactions are selected from the group consisting of enzymatic cleavage, chemical cleavage, and physical cleavage.

5

18.   The method of claim 17 wherein the complementary cleavage reactions are characterized by a relaxed mono-nucleotide, mono-nucleotide, relaxed di-nucleotide, or di-nucleotide specificity.

10   19.   The method of claim 17 or claim 18 wherein the chemical cleavage reaction consists of an alkali treatment, and the one or more target nucleic acids are RNA transcripts that have incorporated non-canonical substrates with a 2′-substituent other than a hydroxyl group.

15   20.   The method of claim 17 or claim 18 wherein the one or more target nucleic acids are subjected to enzymatic cleavage reaction using one or more enzymes selected from the group consisting of endonucleases and exonucleases.

21.   The method of claim 20 wherein the one or more target nucleic
20 acids are subjected to enzymatic cleavage reaction using one or more endonucleases, selected from the group consisting of restriction enzymes, RNA endonucleases, DNA endonucleases and non-specific phosphodiesterases.

22.   The method of claim 21 wherein the one or more endonucleases
25 are one or more selective or non-selective RNA endonucleases and the one or more target nucleic acids are transcripts.

23.   The method of claim 22 wherein the one or more selective or non-selective RNA endonuclease is selected from the group consisting of the G-specific T_1

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ribonuclease, the A-specific U_2 ribonuclease, the A/U specific phyM ribonuclease, the U/C specific ribonuclease A, the C-specific chicken liver ribonuclease (RNaseCL3), and cusativin.

24.   The method of claim 15 wherein the one or more target nucleic acids are phosphorothioate-modified single-stranded DNA or RNA and the endonuclease is nuclease P1.

25.   The method of claim 1 wherein the mass spectroscopical analysis of the nucleic acid fragments is performed using a mass spectroscopic method selected from the group consisting of Matrix-Assisted Laser Desorption/Ionization-Time-of-flight (MALDI-TOF), Electrospray-Ionization (ESI), and Fourier Transform-Ion Cyclotron Resonance (FT-ICR).

26.   A method for scoring known nucleotide sequence variations of one or more target nucleic acids for which a known reference nucleic acid sequence is available, the method comprising the steps of:

(a)   deriving from one or more biological samples the one or more target nucleic acids;

(b)   subjecting the one or more target nucleic acids to complementary cleavage reactions with one or more cleavage reagents thereby generating cleavage products;

(c)   performing mass spectroscopical analysis on the cleavage products obtained in step (b) to obtain two or more mass spectra; and

(d)   comparing the one or more mass spectra of the cleavage products of the one or more target nucleic acids obtained in step c) with the known or predicted mass spectra for the reference nucleic acid sequence, and scoring therefrom, by systematic computational analysis the presence or absence of the known nucleotide sequence variations of the one or more target nucleic acids.

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27.   The method of claim 26 wherein the nucleic acid sequence variation of the target nucleic acid scored in step d) is a deletion, a substitution, insertion or combinations thereof.

28.   The method of claim 26 wherein the one or more target nucleic acids are derived from biological samples selected from the group consisting of an eukaryote, a prokaryote, and a virus.

29.   The method of claim 26 or claim 27 wherein the one or more target nucleic acids are selected from the group consisting of a single stranded DNA, a double stranded DNA, a single stranded RNA, a double stranded RNA, and a DNA/RNA hybrid.

30.   The method of claim 29 wherein the one or more target nucleic acids are derived by one or more consecutive amplification procedures selected from the group consisting of *in vivo* cloning, the polymerase chain reaction (PCR), reverse transcription followed by the polymerase chain reaction (RT-PCR), strand displacement amplification (SDA), and transcription based processes.

31.   The method of claim 29 wherein the one or more amplified target nucleic acids are RNA transcripts generated by a process comprising the steps of:

        (a)   linking operatively a transcription control sequences to the one or more target nucleic acids; and

        (b)   transcribing one or both strands of the one or more target nucleic acids using one or more RNA polymerases that recognize the transcription control sequence on the one or more target nucleic acids.

32.   The method of claim 31 wherein the transcription control sequence

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is selected from the group consisting of an eukaryotic transcription control sequence, a prokaryotic transcription control sequence, and a viral transcription control sequence.

33.   The method of claim 32 wherein the prokaryotic transcription control sequence is selected from the group consisting of T3, T7, or SP6 promoters.

34.   The method of claim 33 wherein the RNA polymerases which utilize the T3, T7 or SP6 promoters are either wild type or in a mutant form capable of incorporating non-canonical substrates with a 2'-substituent other than a hydroxyl group.

35.   The method of claim 30, 31 or 32 wherein the one or more target nucleic acids are derived using a modified nucleoside triphosphates selected from the group consisting of mass modified deoxynucleoside triphosphates, mass modified dideoxynucleoside triphosphates, and mass modified ribonucleoside riphosphates.

36.   The method of claim 35 wherein the modified nucleoside triphosphates are modified on the base, sugar, and/or the phosphate moiety, and are introduced through an enzymatic step, chemically, or a combination of both.

37.   The method of claim 35 wherein the modification consists of 2'-substituents other than a hydroxyl group on the nucleoside triphosphates.

38.   The method of claim 35 wherein the modification consists of phosphorothioate internucleoside linkages or phosphorothioate internucleoside linkages further reacted with an alkylating reagent.

39.   The method of claim 35 wherein the modification consists of a methyl group on C5 of the uridine-5'-monophosphate subunits.

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40.   The method of claim 35 wherein the modifications alter the specificity of cleavage by the one or more cleavage reagents and/or the mass and/or the length of the cleavage products.

41.   The method of claim 36, 37, 38, 39 or 40 wherein the modifications alter the specificity of cleavage by the one or more cleavage reagents and/or the mass and/or the length of the cleavage products.

42.   The method of claim 26 wherein the complementary cleavage reactions are from the group consisting of enzymatic cleavage, chemical cleavage, and physical cleavage.

43.   The method of claim 42 wherein the complementary cleavage reactions are characterized by a relaxed mono-nucleotide, mono-nucleotide, relaxed di-nucleotide, or di-nucleotide specificity.

44.   The method of claim 42 or claim 43 wherein the chemical cleavage consists of an alkali treatment, and the one or more target nucleic acids are RNA transcripts that incorporate non-canonical substrates with a 2′-substituent other than a hydroxyl group.

45.   The method of claim 42 or claim 43 wherein the one or more target nucleic acids are subjected to enzymatic cleavage reaction using one or more enzymes selected from the group consisting of endonucleases and exonucleases.

46.   The method of claim 45 wherein the one or more target nucleic acids are subjected to enzymatic cleavage reaction using one or more endonucleases, selected from the group consisting of restriction enzymes, RNA endonucleases, DNA

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endonucleases, and non-specific phosphodiesterases.

47.   The method of claim 46 wherein the one or more endonucleases are the one or more selective or non-selective RNA endonucleases and one or more target nucleic acids are transcripts.

48.   The method of claim 46 or claim 47 wherein the one or more RNA endonucleases are selected from the group consisting of the G-specific T_1 ribonuclease, the A-specific U_2 ribonuclease, the A/U specific phyM ribonuclease, the U/C specific ribonuclease A, the C-specific chicken liver ribonuclease (RNaseCL3), and cusativin.

49.   The method of claim or claim wherein the one or more target nucleic acids are phosphorothioate- modified single-stranded DNA or RNA and the endonuclease is nuclease P1.

50.   The method of claim 26 wherein the mass spectroscopical analysis of the nucleic acid fragments is performed using a mass spectroscopic method selected from the group consisting of Matrix-Assisted Laser Desorption/Ionization-Time-of-flight (MALDI-TOF), Electrospray-Ionization (ESI), and Fourier Transform-Ion Cyclotron Resonance (FT-ICR).

51.   A method for determining the sequence of one or more target nucleic acids, the method comprising the steps of:

(a)   deriving from a biological sample material the one or more target nucleic acids;

(b)   subjecting the one or more target nucleic acids to complementary cleavage reactions with one or more cleavage reagents thereby generating cleavage products;

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(c)   performing mass spectroscopical analysis on the cleavage products obtained in step (b) to obtain a mass spectra of the nucleic acid fragments; and

(d)   deducing therefrom the nucleotide sequence of the one or more target nucleic acids by systematic computational analysis on the mass spectra of the cleavage products obtained in step (c).

52.   A method for genome wide genotyping of one or more known or unknown target nucleic acids, the method comprising the steps of:

(a)   deriving from one or more biological samples the one or more target nucleic acids;

(b)   subjecting the one or more target nucleic acids to one or more specific cleavage reactions with one or more cleavage reagents thereby generating cleavage products;

(c)   performing mass spectroscopical analysis on the cleavage products obtained in step (b) to obtain one or more mass spectra; and

(d)   comparing the mass spectra of the cleavage products obtained in step c) with reference mass spectra, and diagnosing therefrom genetically relevant nucleic acid sequence variations of the one or more known or unknown target nucleic acids.

53.   A method for identifying one or more target nucleic acids in one or more biological samples, the method comprising the steps of:

(a)   deriving from one or more biological samples the one or more target nucleic acids;

(b)   subjecting the one or more target nucleic acids of step (a) to one or more specific cleavage reactions with one or more cleavage reagents thereby generating cleavage products;

(c)   performing mass spectroscopical analysis on the cleavage

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products obtained in step (d) to obtain one or more mass spectra; and

(d)   comparing the one or more mass spectra obtained in step c) with each other or with a plurality of mass spectra of reference nucleic acids, and deducing therefrom the identity of the one or more target nucleic acids.

54.   The method of claim 53 wherein the one or more target nucleic acids are cDNA.

55.   The method of claim 54 wherein the method is used to determine the expression profile in a biological sample.

56.   A kit for sequence analysis of one or more target nucleic acids using mass spectroscopy, the kit comprising:

(a)   one or more sets of reference nucleic acids for which the nucleic acid sequence is known;

(b)   one or more nucleotide triphosphates;

(c)   one or more polymerases;

(d)   one or more nucleic acid cleaving agents; and

(e)   computer software for comparing the mass spectra of the one or more target nucleic acid with the mass spectra of the reference nucleic acid and deducing therefrom the nucleic acid sequence of the target nucleic acid.

(54) Title: DIAGNOSTIC SEQUENCING BY A COMBINATION OF SPECIFIC CLEAVAGE AND MASS SPECTROMETRY

(57) Abstract

The present invention is in the field of nucleic acid–based diagnostic assays. More particularly, it relates to methods useful for the "diagnostic sequencing" of regions of sample nucleic acids for which a prototypic or reference sequence is already available (also referred to as "re-sequencing"), or which may be determined using the methods described herein. This diagnostic technology is useful in areas that require such re–sequencing in a rapid and reliable way: (i) the identification of the various allelic sequences of a certain region/gene, (ii) the scoring of disease–associated mutations, (iii) the detection of somatic variations, (iv) studies in the field of molecular evolution, (v) the determination of the nucleic acid sequences of prokaryotic and eukaryotic genomes; (vi) identifying one or more nucleic acids in one or more biological samples; (vii) and determining the expression profile of genes in a biological sample and other areas.

FIGURE 1A

FIGURE 1B

FIGURE 2

4/19
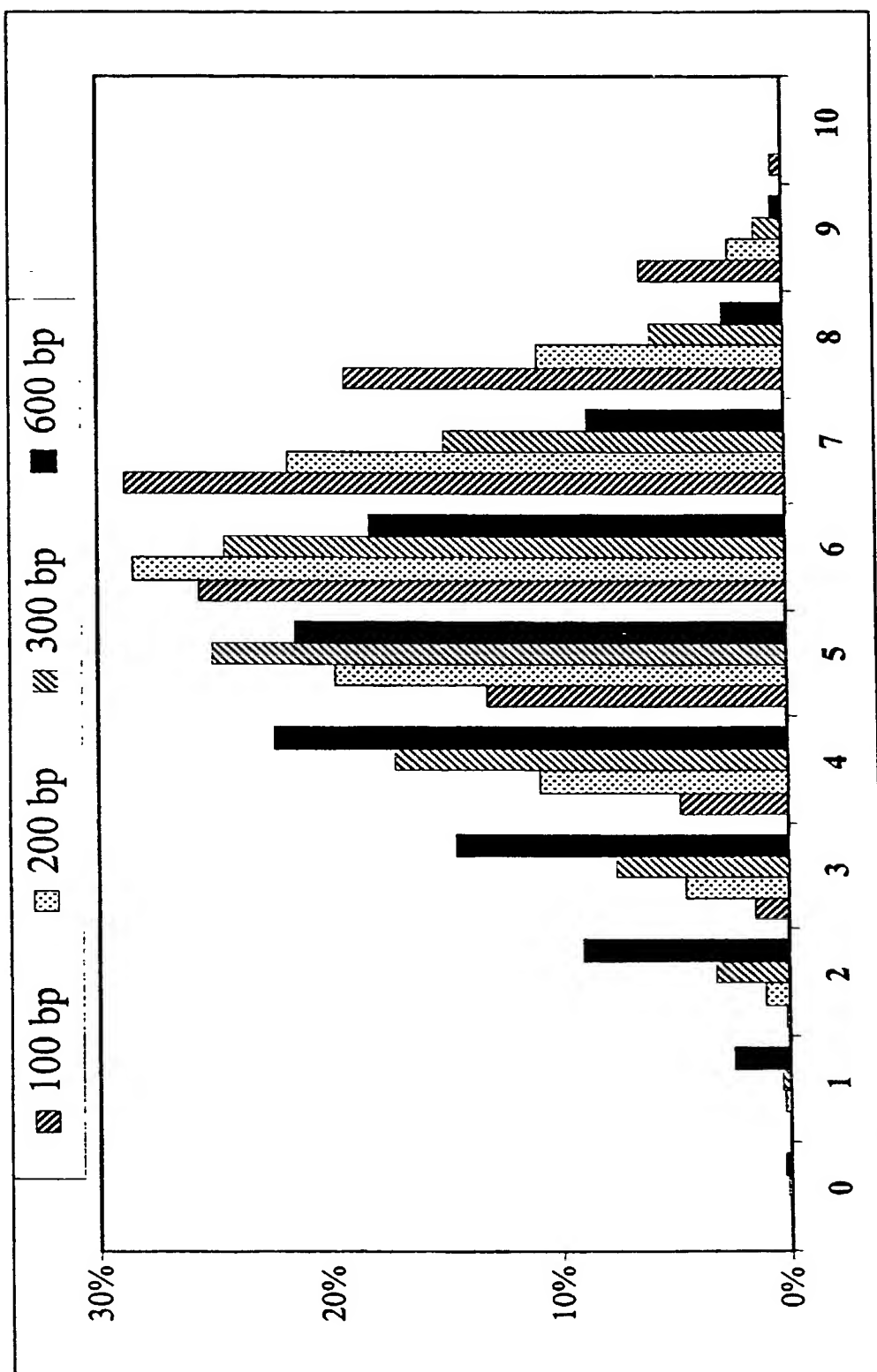
FIGURE 3

10/018453
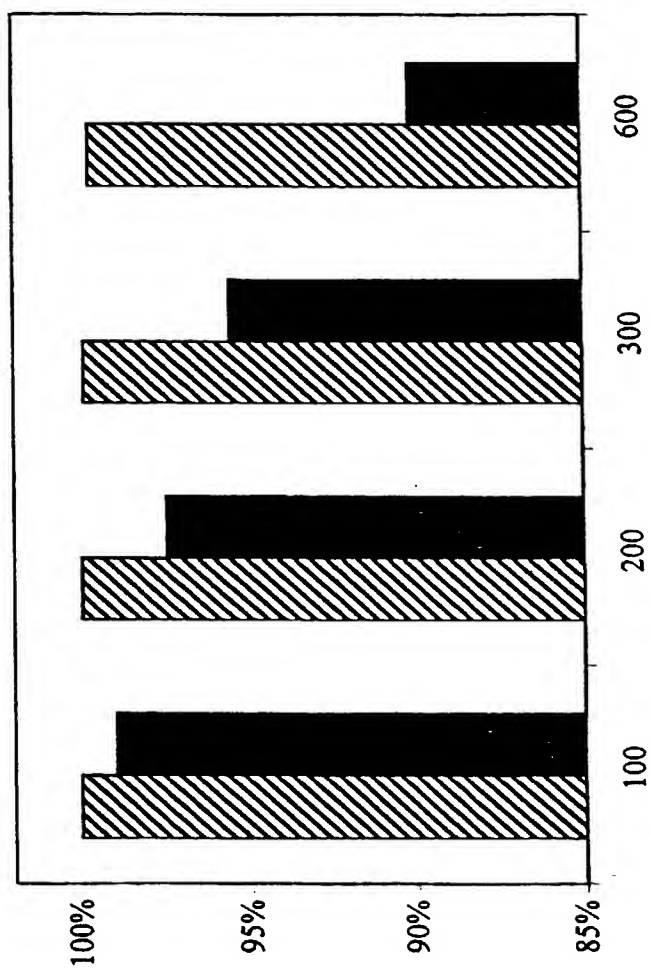
FIGURE 4

```
     GTAAAACGAC GGCCAGTGAA TTGTAATACG ACTCACTATA  (SEQ ID NO: 2)
-->
GGGCGAATTC GAGCTCGGTA CCCGGGGATC CTCTAGAGTC GACCTGCAGG
CATGCAAGCT TGAGTATTCT ATAGTGTCAC CTAAATAGCT TGGCGTAATC  100
ATGGTCATAG CTGTTTCCTG TGTGAAATTG TTATCCGCTC ACAATTCCAC  (118)
ACAACATACG AGCCGGAAGC ATAAAGTGTA AAGCCTGGGG TGCCTAATGA  200
GTGAGCTAAC TCACATTAAT TGCGTTGCGC TCACTGCCCG CTTTCCAGTC
GGGAAACCTG TCGTGCCAGC TGCATTAATG AATCGGCCAA CGCGCGGGGA  300
GAGGCGGTTT GCGTATTGGG CGCTCTTCCG CTTCCTCGCT CACTGACTCG
CTGCGCTCGG TCGTTCGGCT GCGGCGAGCG GTATCAGCTC ACTCAAAGGC  400
GGTAATACGG TTATCCACAG AATCAGGGGA TAACGCAGGA AAGAACATGT
GAGCAAAAGG CCAGCAAAAG GCCAGGAACC GTAAAAAGGC CGCGTTGCTG  500
GCGTTTTTCC ATAGGCTCCG CCCCCCTGAC GAGCATCACA AAAATCGACG
CTCAAGTCAG AGGTGGCGAA ACCCGACAGG ACTATAAAGA TACCAGGCGT  600
TTCCCCCTGG AAGCTCCCTC GTGCGCTCTC CTGTTCCGAC CCTGCCGCTT
ACCGGATACC TGTCCGCCTT TCTCCCTTCG GGAAGCGTGG CGCTTTCTCA  700
TAGCTCACGC TGTAGGTATC TCAGTTCGGT GTAGGTCGTT CGCTCCAAGC
TGGGCTGTGT GCACGAACCC CCCGTTCAGC CCGACCGCTG CGCCTTATCC  800
GGTAACTATC GTCTTGAGTC CAACCCGGTA AGACACGACT TATCGCCACT
GGCAGCAGCC ACTGGTAACA GGATTAGCAG AGCGAGGTAT GTAGGCGGTG  900
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GTATTTGGTA TCTGCGCTCT GC (SEQ ID NO: 3)              (972)
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FIGURE 5
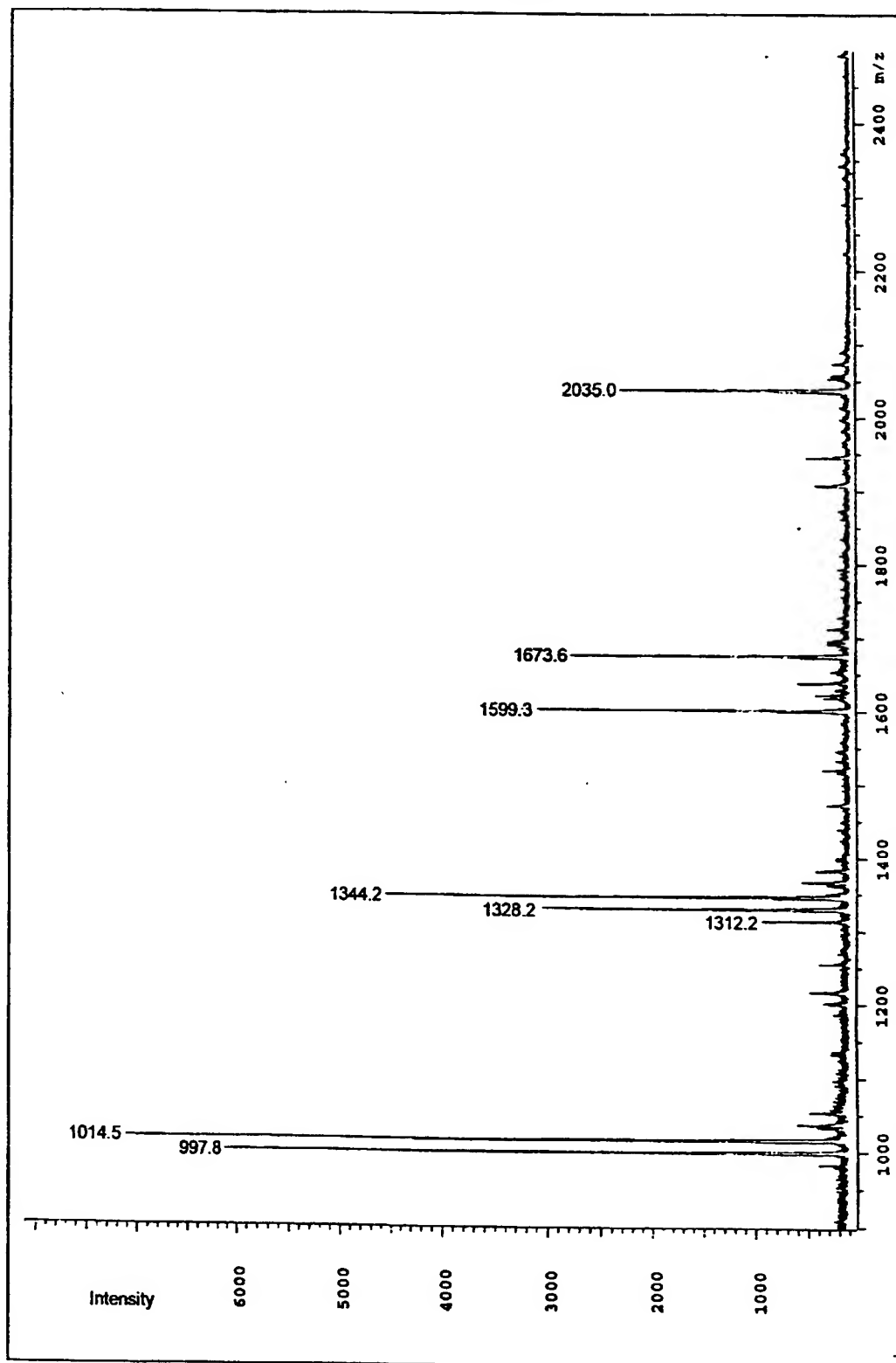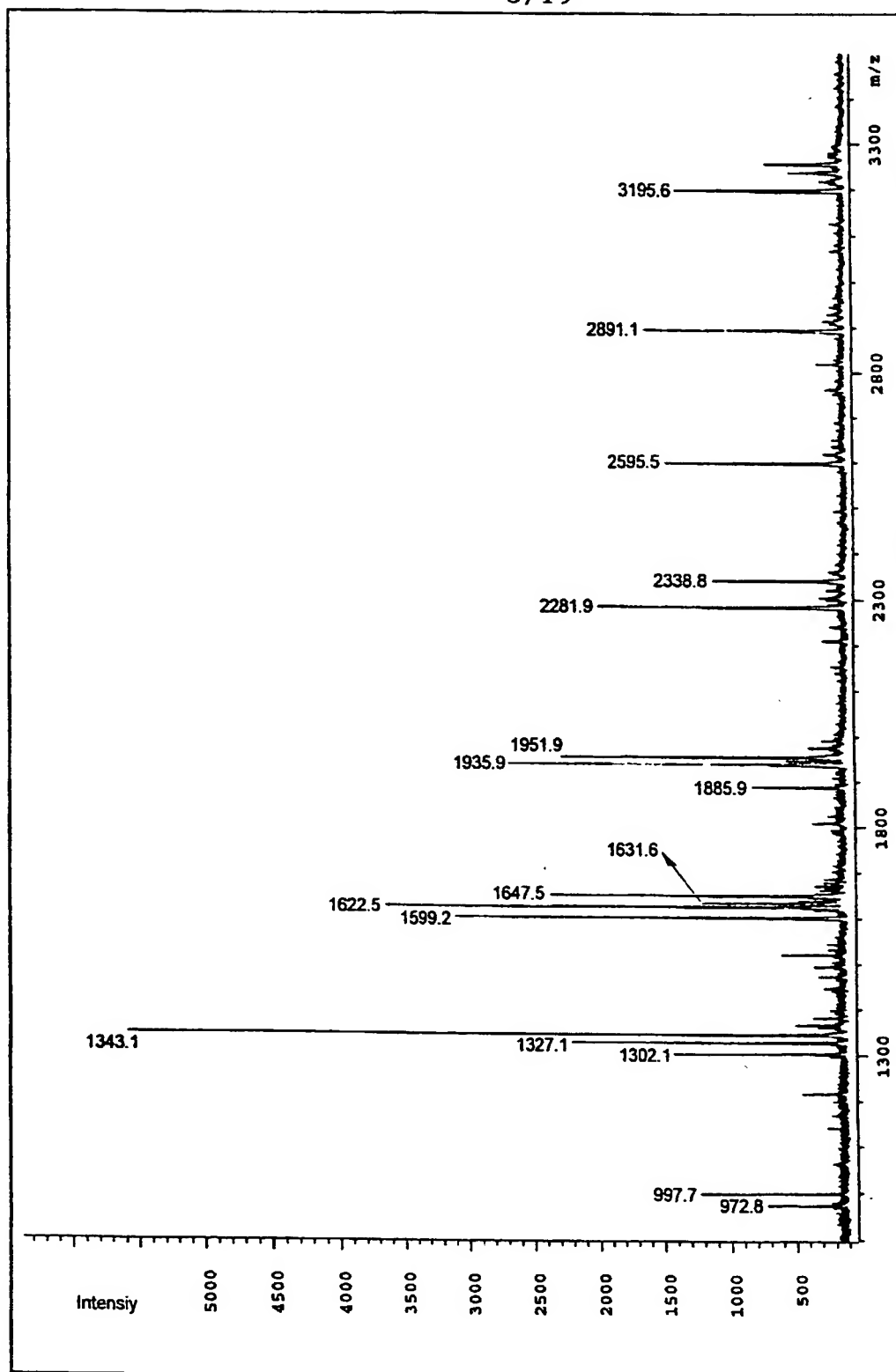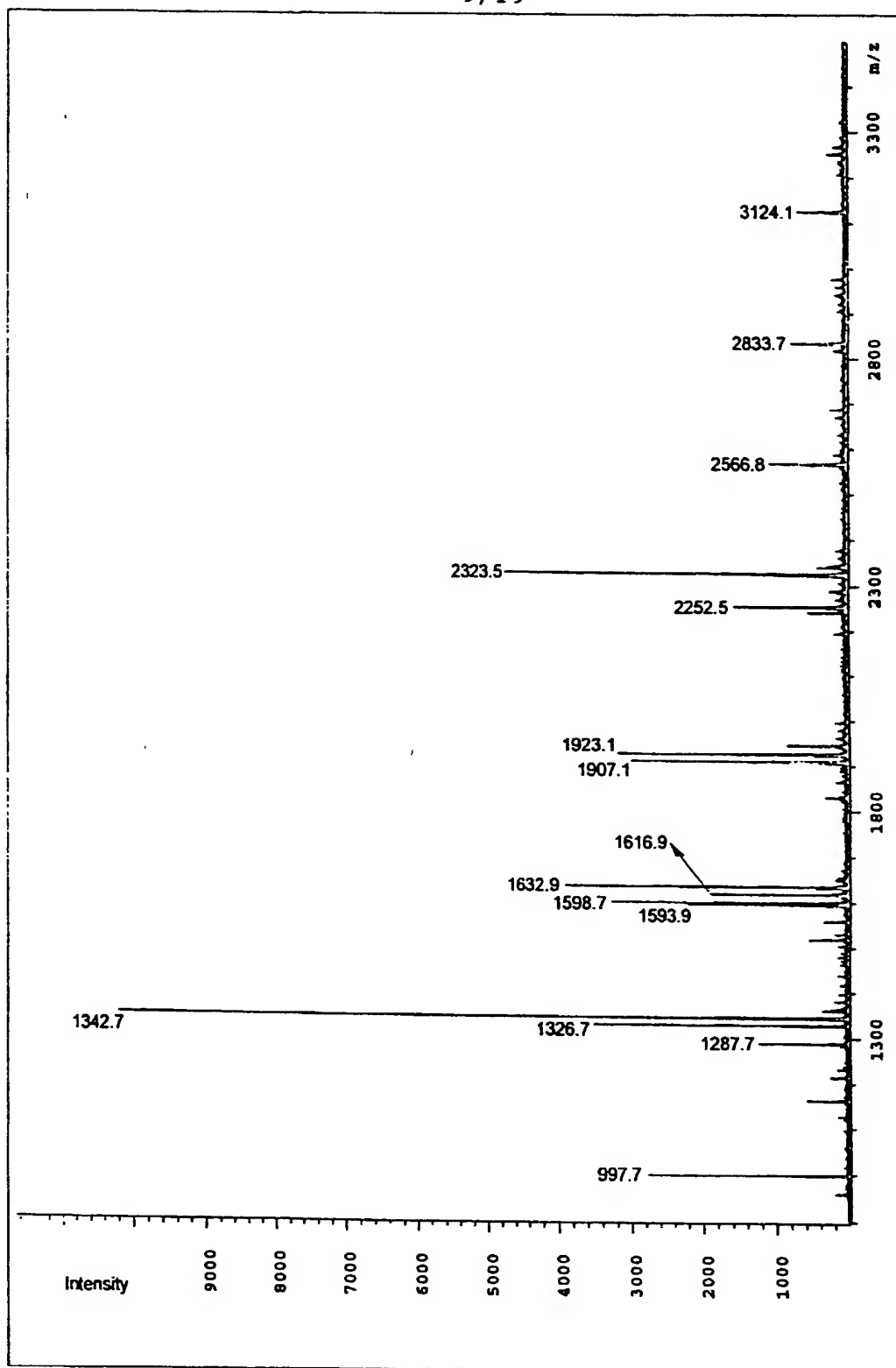
FIGURE 6A
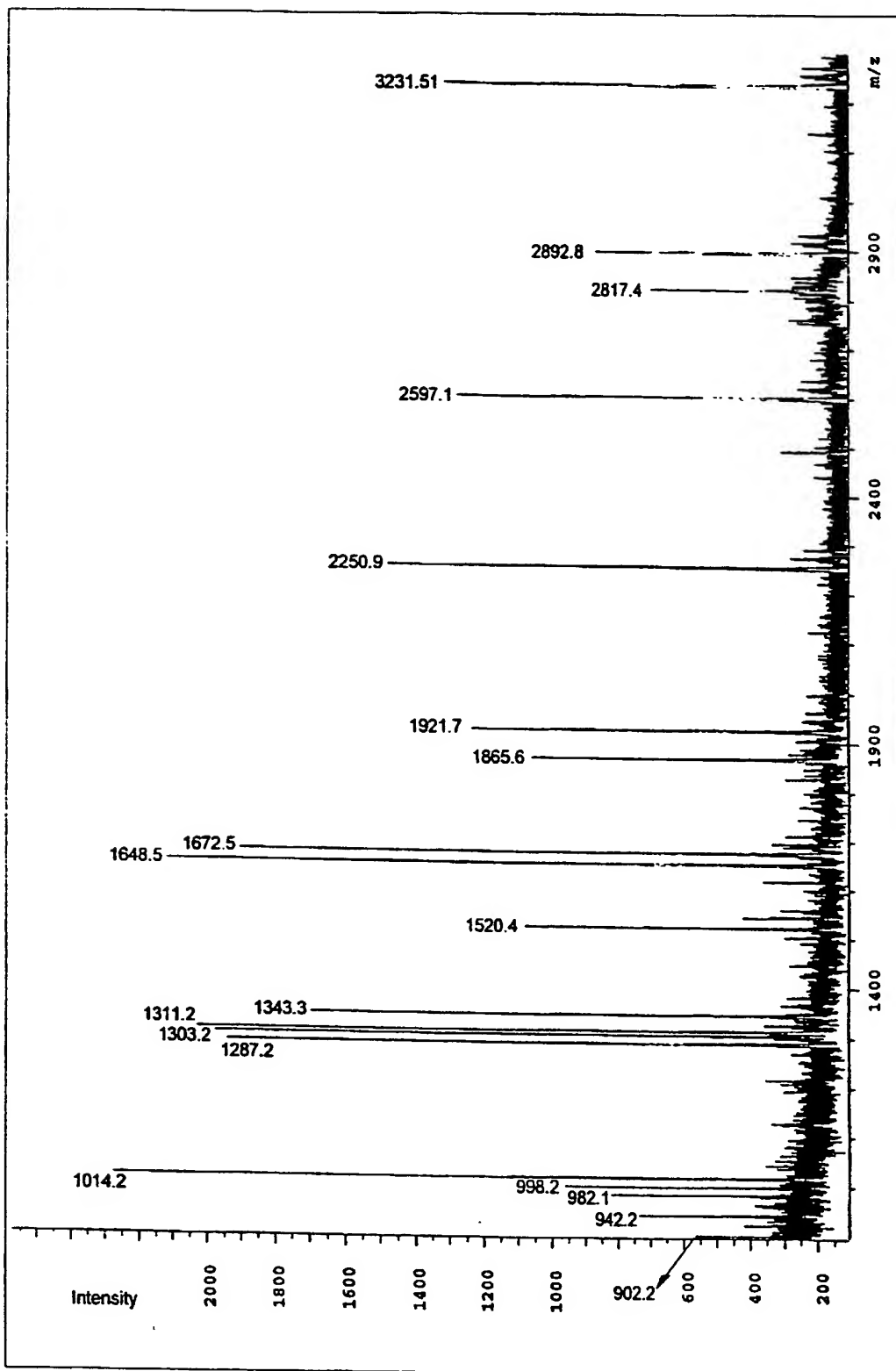
FIGURE 6B

FIGURE 6C

FIGURE 6D

TAATACGACT CACTATAGGG CGACTTCACG AAGACGGTGA AACTGTTGGA TCCAATTCTT ACCCACACAA

ATACAACAAC TACGAAGGTT TTGATTTCTC TGTGAGCTCT CCCTACTACG AATGGCCTAT C (SEQ ID NO: 4)

TAATACGACT CACTATAGGG CGAATTCGTA GTAGGGAGAG CTCACAGAGA AATCAAAACC TTCGTAGTTG

TTGTATTTGT GTGGGTAAGA ATTGGATCCA ACAGTTTCAC CGTCTTCGTG AAGTTTATAT CCGG (SEQ ID NO: 5)

FIGURE 7A

reference   GGA.TCC.AAT.TCT.TAC.CCA.CAC.AAA.TAC.AAC.AAC.TAC.GAA.GGT.TTT (SEQ ID NO: 6)

mutant #1   GGA.TCC.AAT.TCT.TAC.T⬛C.CCA.CAC.AAA.TAC.AAC.AAC.TAC.GAA.GGT.TTT (SEQ ID NO: 7)

mutant #2   GGA.TCC.AAT.TCT.TAC.CCA.CAC.AAA.TAC.AAC.AA⬛.TAC.GAA.GGT.TTT (SEQ ID NO: 8)

mutant #3   GGA.TCC.AAT.TCT.TAC.CCA.CAC.AAA.TAC.AAC.AAC.TAC.GA⬛.GGT.TTT (SEQ ID NO: 9)

mutant #4   GGA.TCC.AAT.TCT.TAC.CCA.CAC.AAA.TAC.AAC.AAC.TAC.G⬛A.GGT.TTT (SEQ ID NO: 10)

mutant #5   GGA.TCC.AAT.TCT.TAC.CCA.CAC.AAA.TAC.AAC.A⬛⬛.TAC.GAA.GGT.TTT (SEQ ID NO: 11)

mutant #6   GGA.TCC.AAT.TCT.TAC.CCA.CAC.⬛A⬛.TAC.AAC.AAC.TAC.GAA.GGT.TTT (SEQ ID NO: 12)

mutant #7   GGA.TCC.⬛⬛⬛.TCT.TAC.CCA.CAC.AAA.TAC.AAC.AAC.TAC.GAA.GGT.TTT (SEQ ID NO: 13)

mutant #8   GGA.TCC.AAT.TCT.TAC.CCA.CAC.AAA.TAC.AA⬛.AA⬛.T⬛C.GAA.GGT.TTT (SEQ ID NO: 14)

FIGURE 7B
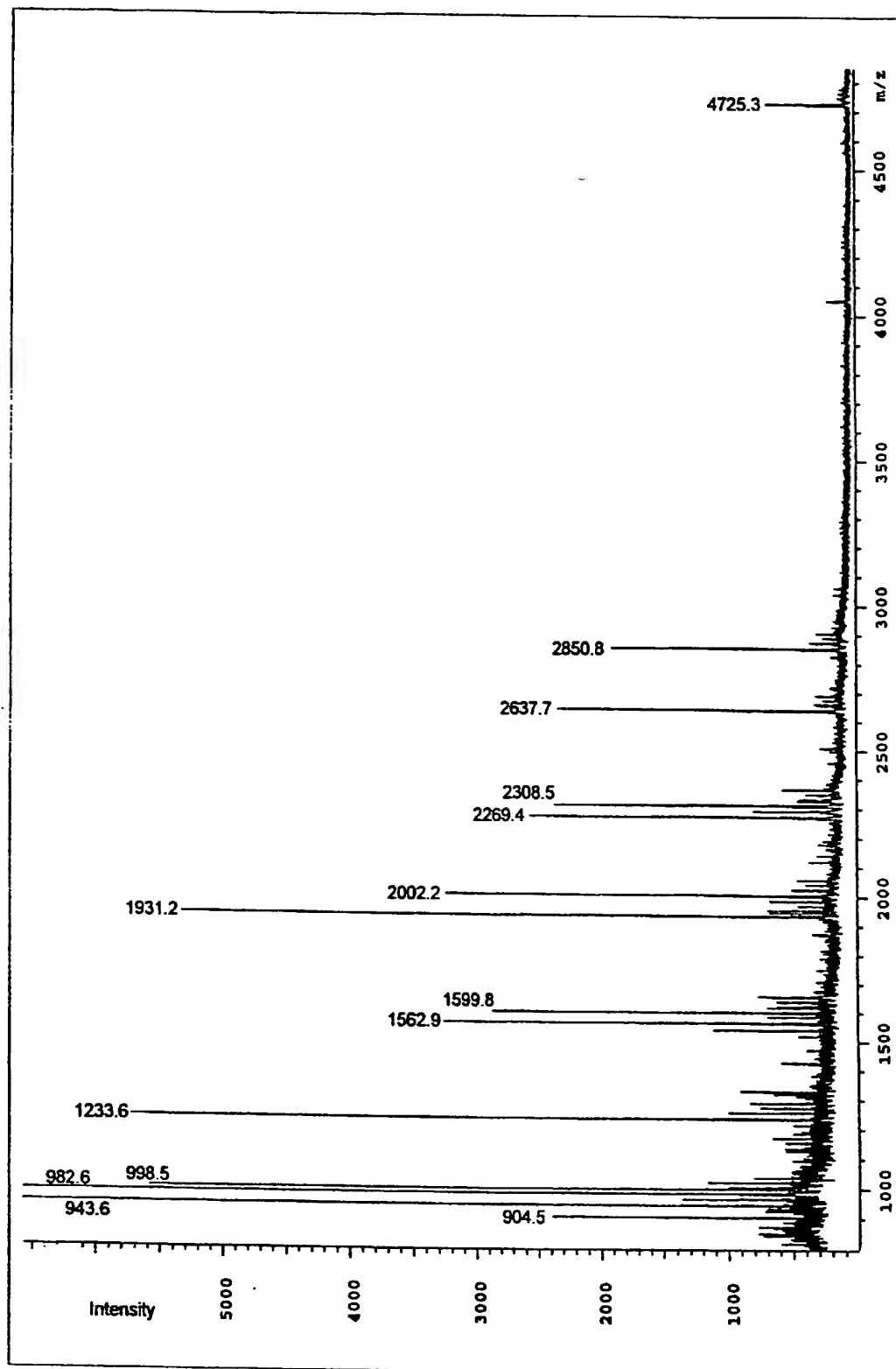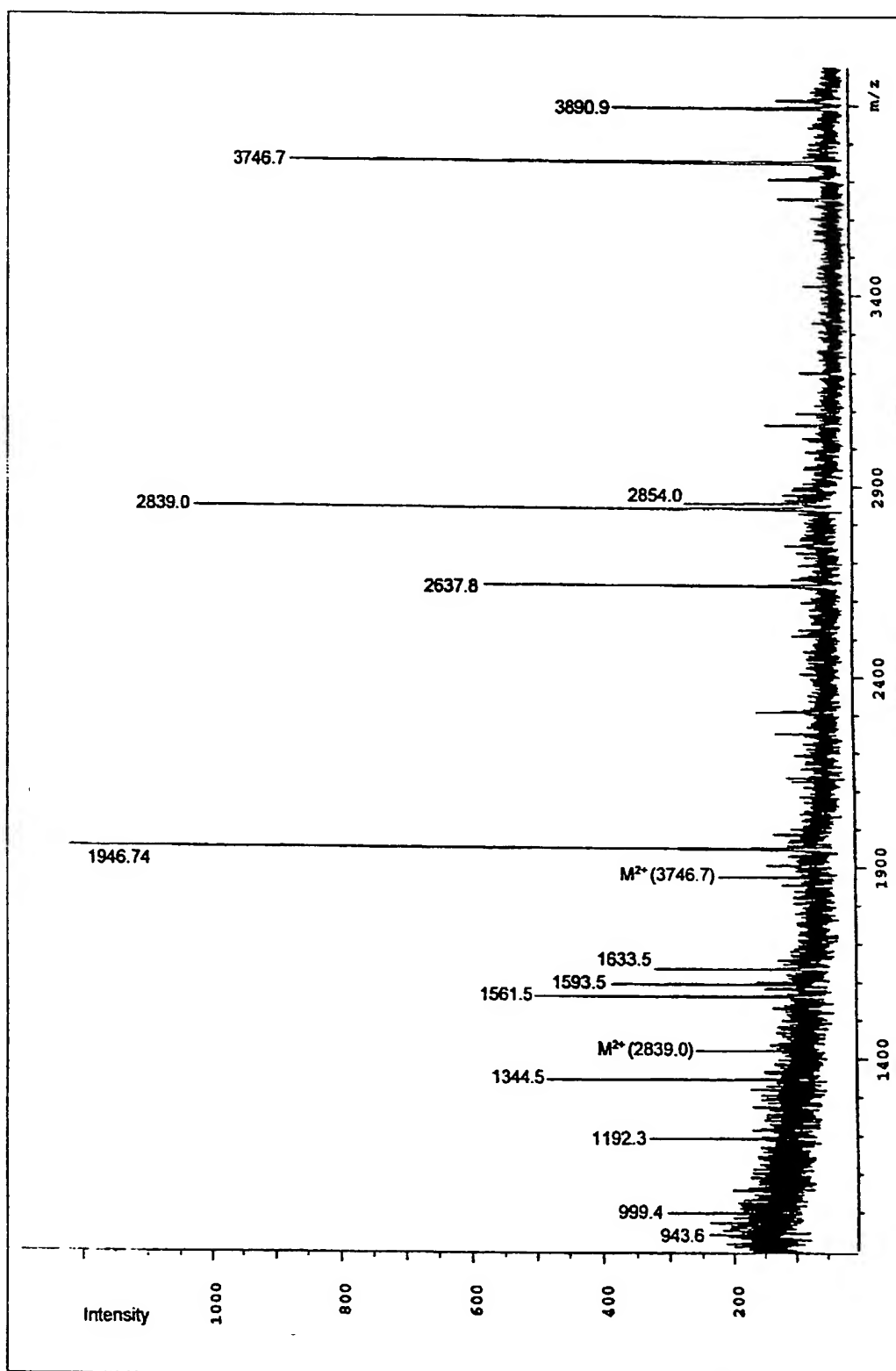
FIGURE 8A

FIGURE 8B

FIGURE 8C

FIGURE 8D

FIGURE 9A

100118453.103001

Intensity

1000
900
800
700
600
500
400
300
200
100

m/z

2446.8

2400

2325.0
2308.9
2293.0
2284.9

2252 9

2237.9

2212.9

2200

2195.8

2172.8

2156.8

1987.7

2000

1939.6

1923.6

1891.6

1883.6

1876.5

1827.6

FIGURE 9B

FIGURE 9C

DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name; I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled **"Diagnostic sequencing by a combination of specific cleavage and mass spectrometry"** the specification of which was filed as International Application No. PCT/EP00/03904 on 30 April 2000, which claimed priority to U.S. Provisional Application No. 60/131,984, filed on 30 April 1999. The text of International Application No. PCT/EP00/03904 is attached herewith I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment(s) referred to above I acknowledge the duty to disclose to the Patent and Trademark Office all information known to me to be material to patentability as defined in 37 C.F.R. §1.56.

I hereby claim foreign priority benefits under 35 U.S.C. §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

Priority Claimed
☐ Yes ☐ No

(Application Serial Number)	(Country)	(Day/Month/Year Filed)

☐ Yes ☐ No

(Application Serial Number)	(Country)	(Day/Month/Year Filed)

I hereby claim the benefit under 35 U.S C. §119(e) of any United States provisional application(s) listed below:

60/131,984   30 April, 1999
(Application Serial Number)   (Day/Month/Year Filed)

(Application Serial Number)   (Day/Month/Year Filed)

I hereby claim the benefit under 35 U.S.C. §120 of any United States application(s) or PCT international application(s) designating the United States of America listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior application(s) in the manner provided by the first paragraph of 35 U.S.C. §112, I acknowledge the duty to disclose to the Office all information known to me to be material to patentability as defined in 37 C.F.R. §1.56 which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application·

(Application Serial Number)	(Day/Month/Year Filed)	(Status-Patented, Pending or Abandoned)

(Application Serial Number)	(Day/Month/Year Filed)	(Status-Patented, Pending or Abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. §1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: I hereby appoint as my attorneys, with full powers of substitution and revocation to prosecute this application and transact all business in the Patent and Trademark Office connected therewith:

(23)

John B. Lungmus (18,566)	Richard H. Anderson (26,526)	Martin J. Hirsch (32,237)	Roger A. Heppermann (37,641)
Allen H. Gerstein (22,218)	Patrick D. Ertel (26,877)	James J Napoli (32,361)	David A Gass (38,153)
Nate F. Scarpelli (22,320)	Richard B. Hoffman (26,910)	Richard M. La Barge (32,254)	Gregory C. Mayer (38,238)
Michael F. Borun (25,447)	James P. Zeller (28,491)	Robert M Gerstein (34,824)	Michael R Weiner (38,359)
Trevor B. Joike (25,542)	Kevin D Hogg (31,839)	Anthony G. Sitko (36,278)	William K. Merkel (40,725)
Carl E. Moore, Jr. (26,487)	Jeffrey S Sharp (31,879)	James A Flight (37,622)	
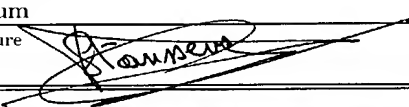
Send correspondence to: Nabeela R. McMillian

FIRM NAME	PHONE NO	STREET	CITY & STATE	ZIP CODE
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1-U

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State or Country: Belgium	State or Country: Belgium
Date ☒ September 28, 2001	Signature ☒ *(signature)*

2-U

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State or Country: Belgium	State or Country: Belgium
Date ☒ September 28, 2001	Signature ☒ *(signature)*

Third Joint Inventor, if any	Citizenship
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City (Zip)	City (Zip)
State or Country	State or Country
Date ☒	Signature ☒

Fourth Joint Inventor, if any	Citizenship
Residence Address – Street	Post Office Address – Street
City (Zip)	City (Zip)
State or Country	State or Country
Date ☒	Signature ☒

37 CFR 1.56. DUTY OF DISCLOSURE - INFORMATION MATERIAL TO PATENTABILITY (Applicable Portion)

(a) A patent by its very nature is affected with a public interest. The public interest is best served, and the most effective patent examination occurs when, at the time an application is being examined, the Office is aware of and evaluates the teachings of all information material to patentability. Each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is canceled or withdrawn from consideration, or the application becomes abandoned. Information material to the patentability of a claim that is canceled or withdrawn from consideration need not be submitted if the information is not material to the patentability of any claim remaining under consideration in the application. There is no duty to submit information which is not material to the patentability of any existing claim. The duty to disclose all information known to be material to patentability is deemed to be satisfied if all information known to be material to patentability of any claim issued in a patent was cited by the Office or submitted to the Office in the manner prescribed by §§ 1.97(b)–(d) and 1.98. However, no patent will be granted on an application in connection with which fraud on the Office was practiced or attempted or the duty of disclosure was violated through bad faith or intentional misconduct. The Office encourages applicants to carefully examine:

(1)   prior art cited in search reports of a foreign patent office in a counterpart application, and

(2)   the closest information over which individuals associated with the filing or prosecution of a patent application believe any pending claim patentability defines, to make sure that any material information contained therein is disclosed to the Office.

Information relating to the following factual situations enumerated in 35 USC 102 and 103 may be considered material under 37 CFR 1.56(a).

35 U.S.C. 102. CONDITIONS FOR PATENTABILITY: NOVELTY AND LOSS OF RIGHT TO PATENT

A person shall be entitled to a patent unless —

(a) the invention was known or used by others in this country, or patented or described in a printed publication in this or a foreign country, before the invention thereof by the applicant for patent, or

(b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of the application for patent in the United States, or

(c) he has abandoned the invention, or

(d) the invention was first patented or caused to be patented, or was the subject of an inventor's certificate, by the applicant or his legal representatives or assigns in a foreign country prior to the date of the application for patent in this country on an application for patent or inventor's certificate filed more than twelve months before the filing of the application in the United States, or

(e) the invention was described in a patent granted on an application for patent by another filed in the United States before the invention thereof by the applicant for patent, or on an international application by another who has fulfilled the requirements of paragraph (1), (2), and (4) of section 371(c) of this title before the invention thereof by the applicant for patent, or

(f) he did not himself invent the subject matter sought to be patented, or

(g) before the applicant's invention thereof the invention was made in this country by another who had not abandoned, suppressed, or concealed it. In determining priority of invention there shall be considered not only the respective dates of conception and reduction to practice of the invention, but also the reasonable diligence of one who was first to conceive and last to reduce to practice, from a time prior to conception by the other.

35 U.S.C. 103. CONDITIONS FOR PATENTABILITY; NON-OBVIOUS SUBJECT MATTER (Applicable Portion)

A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

Subject matter developed by another person, which qualifies as prior art only under subsection (f) or (g) of section 102 of this title, shall not preclude patentability under this section where the subject matter and the claimed invention were, at the time the invention was made, owned by the same person or subject to an obligation of assignment to the same person.

35 U.S.C. 112. SPECIFICATION (Applicable Portion)

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

SEQUENCE LISTING

<110> METHEXIS NV

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      DIGESTION AND MASS SPECTROMETRY

<130> 29314/35410A

<140>
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<150> 60/131,984
<151> 1999-04-30

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<170> PatentIn Ver. 2.1

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<213> Homo sapiens

<220>
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<211> 40
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<220>
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tgtgaaattg ttatccgctc acaattccac acaacatacg agccggaagc ataaagtgta 180

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ctttccagtc gggaaacctg tcgtgccagc tgcattaatg aatcggccaa cgcgcgggga 300
gaggcggttt gcgtattggg cgctcttccg cttcctcgct cactgactcg ctgcgctcgg 360
tcgttcggct gcggcgagcg gtatcagctc actcaaaggc ggtaatacgg ttatccacag 420
aatcagggga taacgcagga aagaacatgt gagcaaaagg ccagcaaaag gccaggaacc 480
gtaaaaaggc cgcgttgctg gcgtttttcc ataggctccg ccccccctgac gagcatcaca 540
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<220>
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